THE CHROMATIN FACTOR ENHANCER OF POLYCOMB PROMOTES DIFFERENTIATION OF ADULT GERMLINE STEM CELLS AND CYST STEM CELLS IN THE DROSOPHILA TESTIS

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A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland
October 2016

ABSTRACT

Tissue homeostasis requires stem cell balance between proliferation and differentiation as well as efficient repair of DNA damage, a consequence of consecutive cell divisions. Here, we use Drosophila testes as a model system to study how germ cells modulate this balance and DNA damage repair to maintain genome integrity inherited by the next generation. In testes, Cyclin B (CycB) accumulates in the mitotic region, decreases upon completion of mitosis, and regains robust expression just before meiosis I. Transcriptional and translational repression of CycB in meiotic stages are both well studied. However, the mechanism of CycB downregulation after mitosis and if such downregulation contributes to the transition to differentiation is unknown. Here we find that chromatin factor E(Pc) can repress CycB transcriptionally by modulating H4 acetylation. Moreover, such downregulation specifies four mitotic divisions and is essential to initiate differentiation. Besides regulating this critical transition of germ cells, E(Pc) also functions in the repair of DNA double-strand breaks (DSB). Failure of DSB repair upon E(Pc) depletion results in the accumulation of DSBs and severe germ cell death. In addition, loss of Tip60, a histone acetyltransferase (HAT), leads to a similar germ cell phenotype, indicating E(Pc) cooperates with Tip60 to function intrinsically in germ cells. Taken together, HAT activity plays a crucial role in maintaining tissue homeostasis through transcriptional regulation, ensuring genome integrity for the next generation by repairing DNA damage during spermatogenesis.

In addition, extrinsic cues from the microenvironment where stem cells reside interact with intrinsic factors within stem cells to maintain their identity and govern their activity. Previous studies on the epigenetic regulation of stem cells mainly focused on

understanding intrinsic regulation. Here we report non-cell-autonomous roles of E(Pc) in regulating germ cell differentiation in the *Drosophila* adult testis. E(Pc) acts in the cyst stem cell (CySC) lineage to negatively control the transcription of genes associated with multiple cell signaling pathways, including both the JAK-STAT and the EGF signaling pathways, to promote the differentiation of CySCs. However, inactivation of E(Pc) specifically in the CySC lineage leads to defects in both germline differentiation and the maintenance of germ cell identity, revealing its non-cell-autonomous role in regulating germ cells. Furthermore, compromising the Tip60 histone acetyltransferase activity in the CySC lineage recapitulates loss-of-function phenotypes of E(Pc), suggesting that they act together, consistent with published biochemical data. In summary, these data demonstrate that E(Pc) plays a central role in coordinating differentiation between CySC and germline stem cell lineage.

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Chapter 1

Introduction of epigenetic regulation of germline stem cell lineage

This chapter is based on a previously published review article (Feng and Chen, 2015).

Asymmetric stem cell divisions and *Drosophila* spermatogenesis

Stem cells are unique in their ability to both self-renew and give rise to a variety of differentiated cell types. Adult stem cells are naturally existing cell populations that maintain tissues, such as blood, intestine, muscle, skin, and the germline. Many of them undergo asymmetric cell division to generate a self-renewed stem cell and a daughter cell which will subsequently enter differentiation (Betschinger and Knoblich, 2004; Clevers, 2005; Inaba and Yamashita, 2012; Morrison and Kimble, 2006). Normal activities of adult stem cells are required for homeostasis, tissue regeneration, and fertility. The misdetermination of stem cell fate or the malfunction of stem cell derivatives are common causes of human diseases, such as diabetes, muscular dystrophy, neurodegenerative disease, infertility, and many forms of cancer (Feinberg et al., 2006; Morrison and Kimble, 2006; Rando, 2006; Rossi et al., 2008). It is of particular interest to understand the molecular circuitries underlying the unique features of adult stem cells.

The *Drosophila* male and female germline stem cells (GSCs) are among the best characterized adult stem cell systems in terms of their physiological locations, microenvironments or niches, cellular structures, and the signaling pathways that maintain their stem cell identities (Fuller and Spradling, 2007; Losick et al., 2011; Morrison and Spradling, 2008). Like many other types of adult stem cells [see (Inaba and Yamashita, 2012; Knoblich, 2008; Morrison and Kimble, 2006) for reviews on asymmetric cell division], GSCs undergo asymmetric cell division (ACD) to balance self-renewal and differentiation for tissue homeostasis. Taking the male GSC as an example, the ACD outcome is ensured by both extrinsic cues in the niche and intrinsic factors in

the GSC (Fuller and Spradling, 2007; Yamashita and Fuller, 2005; Yamashita et al., 2005). One critical extrinsic cue for male GSC is the Unpaired (Upd) ligand for the JAK-STAT signaling pathway. Upd emanates from a group of post-mitotic cells at the tip of the fly testis, constituting a "hub" structure (Kiger et al., 2001; Toledano et al., 2012; Tulina and Matunis, 2001). Activated JAK-STAT signaling is required for maintaining GSCs, which are attached to hub cells by adherens junctions (Inaba et al., 2010; Kiger et al., 2001; Leatherman and Dinardo, 2008, 2010; Tulina and Matunis, 2001; Yamashita et al., 2003). In addition to extrinsic signals, intrinsic factors inside male GSCs also contribute to the proper ACD of GSCs. For example, male GSCs always retain the mother centrosome that is anchored at the GSC-hub interface, while the newly synthesized daughter centrosome migrates to the opposite end of GSC. This asymmetric segregation of centrosomes serves as an important intrinsic mechanism to set up proper spindle orientation for ACD of GSCs (Yamashita et al., 2003; Yamashita et al., 2007). Interestingly, in GSCs where the centrosomes are misoriented, the mitotic spindle does not form, therefore GSCs are arrested and cannot proceed with mitosis. This phenomenon has promoted the "centrosome orientation checkpoint" hypothesis as an intrinsic mechanism to ensure ACD (Cheng et al., 2008a; Inaba et al., 2010; Roth et al., 2012; Yuan et al., 2012).

ACD of GSCs initiates spermatogenesis in *Drosophila* testis through both larval and adult stages (**Fig. 1-1**). Localized at the apical tip of testes, post-mitotic hub cells function as a niche to anchor GSCs and cyst stem cells (CySCs). After ACD of GSCs, differentiating gonialblast cells undergo four rounds of synchronous mitosis, a transit amplifying stage, which then progresses to pre-meiotic S phase. This transition requires

the accumulation of the differentiation factor bag-of-marbles (Bam) to a threshold level (Eun et al., 2013; Gonczy et al., 1997; Insco et al., 2009; McKearin and Spradling, 1990; Schulz et al., 2004). Primary spermatocytes resulting from pre-meiotic S phase enter elongated G2 phase, which normally takes 90 hours and gives rise to a 25-fold increase in volume, concomitant with active transcription. Most transcription is shut off upon transit to meiotic divisions (Olivieri and Olivieri, 1965), giving rise to haploid spermatids. These spermatids undergo a dramatic morphological change to differentiate from rounded cells into highly polarized motile sperm for future fertilization.

Epigenetic regulation as an intrinsic mechanism to regulate GSCs In several model organisms, gametogenesis depends on constant GSC functionality (Kimble and Seidel, 2008; Spradling et al.). Like many other types of adult stem cells, GSCs undergo asymmetric cell divisions to give rise to two daughter cells with distinct cell fates: one that self-renews to retain stem cell identity and another one that commits to differentiation. Two daughter cells arise from the same mitotic event and therefore inherit an identical genetic code. Although it is conceivable that the epigenetic information is different in these two cells, the nature of the distinct epigenetic code had not been identified. Using a dual-color labeling strategy to label pre-existing *vs.* newly synthesized canonical histone H3 precisely, our lab has shown that during the asymmetric division of the *Drosophila* male GSC, the preexisting H3 is selectively segregated to the self-renewed GSC daughter cell whereas newly synthesized H3 is enriched in the differentiating daughter cell (Tran et al., 2012) (**Fig. 1-2**). Since post-translational modifications of histones are key epigenetic information, our studies provide the first direct evidence suggesting that the GSC may selectively retain pre-existing histones that

define their stem cell identity whereas the other daughter cell may preferentially reset their chromatin to prepare for differentiation. Another study using CO-FISH (chromosome orientation fluorescence *in situ* hybridization) technique combined with chromosome- and strand-specific probes has shown that sex chromosomes (X and Y) have biased segregation of sister chromatids in the same system (**Fig. 1-2**). Although autosomes do not display such a bias, they show another interesting co-segregation pattern (Yadlapalli and Yamashita, 2013). An earlier paper using nucleoside analog 5-bromo-2-deoxyuridine (BrdU) incorporation assay showed the lack of "an immortal strand" in this system (Yadlapalli et al., 2011). Together, these studies suggest that biased segregation of sister chromatids of sex chromosomes might be due to the different epigenetic information they carry. However, the connection between asymmetric histone inheritance and biased sister chromatid segregation of sex chromosomes remains unclear. It would be interesting to find out whether mutations disrupting one segregation pattern will affect the other pattern.

Even though the histone variant H3.3 is inherited symmetrically at a global level during GSC asymmetric division (Tran et al., 2012), another histone variant H2Av, the *Drosophila* homolog of mammalian H2A.Z/H2A.X, is required for male GSC maintenance. Accordingly, the ATP-dependent chromatin-remodeling factor Domino required for proper H2Av association with DNA also helps to maintain GSC (Morillo Prado et al., 2013). However, H2Av is not required for germ cell differentiation, as several differentiation markers show proper expression in *H2Av* mutant germ cells. Lack of H2Av does not result in global changes in H3K4me3 or H3K27me3 immunostaining; neither does it cause altered Janus kinase signal transducer and activator of transcription

(JAK-STAT) response in GSCs, a critical pathway for GSC maintenance (reviewed by (de Cuevas and Matunis, 2011)). However, it is possible that the *H2Av* mutation disrupts H3K9me2/3-enriched heterochromatin structure in GSCs, as shown in somatic cells previously (Swaminathan et al., 2005). Phosphorylation of H2Av (γH2Av) serves as a specific marker for double-strand DNA breaks which often indicate an early response to DNA damage. Consistently, it was reported that mutations in H2Av enhance *Drosophila* male germline defects caused by DNA damage, resulting from mutations in a Polycomb group (PcG) gene *multi sex combs* (*mxc*) (Landais et al., 2014). Suppression of *mxc* phenotype by a reduction in H3 and enhancement by *H2Av* mutation are not restricted to germline but also apply to somatic cells, suggesting a broader connection between histones and PcG function that may not be mediated through a particular histone modification.

Our understanding of the biological functions of particular histone modifications is primarily derived from studies of mutations that inactivate the particular histone modifying enzyme that 'writes', 'reads', or 'erases' such a modification. For example, *eggless*/*dSETDB1* encodes a histone H3K9 methyltransferase and *egg* mutants show GSC self-renewal defects in *Drosophila* ovary. However, the GSC loss defects are not caused by loss-of-function in the well-known bone morphogenetic protein (BMP) signaling pathway in female GSCs. Therefore these findings suggest a potential H3K9me3-dependent but BMP-independent GSC maintenance mechanism (Wang et al., 2011). Another study on Eggless revealed its function in the transcriptional regulation of piRNA clusters (Rangan et al., 2011). Activated piRNAs act with the PIWI protein to protect germline genome by preventing transposable element activity, which may be a conserved

mechanism for germline genome integrity across species (Huang et al., 2013; Le Thomas et al., 2013; Malone et al., 2009; Rozhkov et al., 2013; Sienski et al., 2012). A few microRNAs have also been found to regulate *Drosophila* female GSC maintenance or differentiation. For example, *miR-184* (Iovino et al., 2009) and *bantam* (Yang et al., 2009) are both required for the balance of GSC maintenance *vs.* differentiation in ovaries. Together, these results strongly suggest GSCs have a unique epigenetic state to help maintain stem cell fate probably by activating and maintaining 'stemness' gene expression while repressing differentiation gene expression.

Extrinsic signaling contributes to neutral competition between CySCs and GSCs

Recent studies identified stem cell competition as a regulatory mechanism to maintain tissue homeostasis (Amoyel et al., 2016; Amoyel et al., 2014; Bondar and Medzhitov, 2010; Issigonis et al., 2009; Jin et al., 2008; Martins et al., 2014; Rhiner et al., 2009; Sheng et al., 2009a; Singh et al., 2016). In Drosophila testis, CySCs competition with each other, as well as neighboring GSCs for niche access has been well studied. Multiple signaling pathways are involved in this stem cell behavior through distinct molecular mechanisms (**Fig. 1-3**). Hh signaling components are enriched in CySCs and required for CySC self-renewal (Amoyel et al., 2013; Amoyel et al., 2014; Michel et al., 2012). The gain of function of Hh signaling activity using *patched* (*ptc*) homozygous clones revealed that *ptc* mutant CySCs can outcompete wild-type GSCs and cause a significant reduction of GSCs (Amoyel et al., 2014). Similar competition and loss of GSCs are also found in CySCs with overexpression of Hh signal transducer Cubitus interruptus (Ci^{Act}). In *ptc* mutant CySCs, no increase in integrin is observed, suggesting adhesion between CySCs and the hub is not enhanced. Interestingly, *ptc* mutant CySCs have a higher

S-phase and M-phase index, suggesting accelerated proliferation is induced with Hh signaling gain of function. Limiting the level of string, the Drosophila homolog of cdc25, reduces the competitive advantage of *ptc* mutant CySCs, indicating the rate of cell proliferation influences CySC competition. Moreover, CySCs with overexpression of Cyclin E, G1/S promoting factor as well as String, G2/M promoting factor, together can outcompete wild-type GSCs and CySCs, demonstrating the sufficiency of proliferation driving CySC competition. In addition to Hh signaling, Hpo signaling, a well-known pathway regulating proliferation (Pan, 2010), also function in CySCs competition.

Besides an increased proliferation rate, integrin also mediates competition between GSCs and CySCs in Drosophila testis (**Fig. 1-3**). Socs36E, a JAK-STAT signaling target, also works as a repressor of this signaling (Callus and Mathey-Prevot, 2002; Karsten et al., 2002), providing a self-regulating, negative feedback loop. *Socs36E* mutant CySCs are able to displace neighboring GSCs in an integrin-dependent manner (Issigonis et al., 2009). A recent study has uncovered the molecular mechanisms regarding how Socs36E regulates integrin expression. The gain of function of JAK-STAT signaling in CySCs exhibit GSC-like tumors and no competition behavior is observed, implying that other signaling regulated by Socs36E might be involved. In addition to JAK-STAT signaling, Socs36E also represses MAPK signaling. *Socs36E* mutant CySCs that are deficient for MAPK signal transduction lose their competitive advance (Amoyel et al., 2016), indicating MAPK signaling is a major regulator governing CySCs competition for niche access. Besides Socs36E, a novel tumor suppressor Mlf1-adaptor molecule (Madm), also negatively regulates MAPK signaling through repressing an EGF ligand, vein, and thus regulates the competition between CySCs and GSCs (Singh et al., 2016). In mice, p53 deficient

hematopoietic stem cells (HSCs) can promote the proliferation of p53 low HSCs, while inducing growth arrest and senescence in p53 high HSCs (Bondar and Medzhitov, 2010). In summary, stem cell competition plays a crucial role to equilibrate two stem cell populations and select for stem cell fitness to prepare for future amplification.

Epigenetic mechanisms cooperate with extrinsic signaling to regulate germline function

Extrinsic cues from the somatic cells in the 'microenvironment' or 'niche' must interplay with intrinsic factors in germ cells to determine their functionality. The somatic cells in gonads could play a passive role in germ cell differentiation. For example, two PcG chromatin regulators Psc and Su(z)2 act in somatic gonadal cells to maintain their identity and restrict them from overproliferation. Loss of their function leads to somatic cell tumors and affect germline function by physically displacing GSCs from their niche (Morillo Prado et al., 2012). Recent studies demonstrate that somatic gonadal cells also play active roles in determining germ cell identity and activity. For example, inactivation of the H3K27me3-specific 'writer' E(z) methyltransferase in somatic gonadal cells causes the neighboring germ cells to adopt somatic cell fate (Eun et al., 2014). This germline cell fate change is restricted to early-stage germ cells including GSCs, suggesting that epigenetic plasticity declines during cellular differentiation. It is conceivable that this non-cell-autonomous regulation of the germline through somatic gonadal cells must act through some signaling pathway(s). Indeed, multiple signaling pathways have been identified for their critical roles for germ cell function, such as JAK-STAT, BMP, epidermal growth factor (EGF) (Matunis et al.), Notch (Kitadate and Kobayashi, 2010), ecdysone (Morris and Spradling), insulin (Hsu and Drummond-

Barbosa) and Slit-Roundabout (Stine et al., 2014) pathways. Interestingly, in many cases signaling pathways act through chromatin regulators. For example, the steroid hormone ecdysone signaling promotes female GSC maintenance through chromatin remodeling factors such as ISWI and Nurf301 acting in GSCs (Ables and Drummond-Barbosa, 2010). A functional interaction between ecdysone hormone signaling and Nurf301 was also seen in *Drosophila* male GSCs, even though in this context the ecdysone signaling acts in somatic cells to regulate germline (Li et al., 2014), suggesting a sex-specific response. Recently, technological advancements have allowed identification of direct target genes in a cell-type-specific manner for both chromatin factors and transcription factors downstream of signaling pathways, which greatly facilitate our understandings of such a crosstalk. For example, the Wnt and EGF pathway genes are identified as direct targets of H3K27me3-methyltransferase E(z) in somatic cells and *egfr* mutants show strong genetic interaction with an *E(z)* mutant in fly testes (Eun et al., 2014). The H3K9me3-methyltransferase Eggless is required in escort cells to regulate *Drosophila* female germ cell differentiation partially through controlling BMP signaling (Wang et al., 2011) (**Fig. 1-4**). Epigenetic 'erasers' such as histone demethylases also regulate germ cells non-cell-autonomously. For example, the H3K27me3-specific demethylase dUTX removes the repressive H3K27me3 mark at the upstream region of the *Socs36E* gene in somatic cells of fly testes. Because Socs36E is an inhibitor of the JAK-STAT pathway, this function of dUTX prevents hyperactivation of JAK-STAT signaling and maintains proper gene expression and architecture of the testis niche and male GSC function (Tarayrah et al., 2013). In *Drosophila* ovary, H3K4me1/2-demethylase Lsd1 function in escort cells on a diverse group genes, including both BMP-related and BMP-unrelated

genes (Eliazer et al., 2014). Therefore, Lsd1 regulates germline differentiation by preventing ectopic BMP signaling outside of the niche (Eliazer et al., 2014; Eliazer et al., 2011) as well as using BMP-independent mechanisms. In addition to histone modifying enzymes, nuclear lamina regulates specific nucleoporin distribution which in turn promotes nuclear localization of phosphorylated ERK, the downstream effector of the EGF pathway (Chen et al., 2013a) (**Fig. 1-4**). This activity of Lamin occurs in somatic gonadal cells but affects the neighboring germline function, which shows how nuclear structure acts as another layer of epigenetic regulation to modulate readout of signaling pathway and regulate germ cell activity.

Epigenetic regulation during germ cell differentiation

Before commitment for meiosis and terminal differentiation, progenitor germ cells normally undergo a transit-amplification stage as mitotic cells. Transit-amplifying (TA) germ cells differ from *bona fide* GSCs in several ways. The progression from GSC to TA germ cells results in loss of asymmetric histone inheritance (Tran et al., 2012) and the potential to turn on somatic-specific transcription factors (Eun et al., 2014). In spite of these differences, at the morphological level TA germ cells have the potential to become GSC-like cells through a dedifferentiation process (reviewed by (Sheng and Matunis, 2009; Yuan and Yamashita, 2010)). However, it remains unclear whether dedifferentiated GSC-like cells recover both molecular and cellular characteristics like *bona fide* GSCs. For example, the well-oriented centrosomes in GSCs are not maintained in dedifferentiated GSC-like cells, leading to a G2/M arrest and declined mitotic activity (Cheng et al., 2008a). More studies are needed to compare molecular features, including

chromatin structure and the transcriptional profile, between GSCs and dedifferentiated germ cells, to fully understand this *in vivo* cellular reprogramming process.

Different from transit-amplifying cells, germ cells cannot dedifferentiate once turning on the meiotic program (Sheng and Matunis, 2011), suggesting fundamental differences of chromatin state between mitotic and meiotic germ cells. High-throughput approaches have been used to identify meiosis-specific histone modifications and interestingly many of them are conserved across species, suggesting potentially common epigenetic mechanisms during meiosis. For example, it is feasible to use yeast sporulation for a genetic screen to identify histone mutants that affect spermatogenesis in higher organisms. This method has led to discoveries of H4S1P and H3T11P as key meiotic modifications in flies and mice (Govin et al., 2010; Krishnamoorthy et al., 2006). Another proteomic approach identified a novel H3K23me3 as another conserved meiosis-specific histone modification from *Tetrahymena* to *C. elegans* (Papazyan et al., 2014).

Intriguingly, meiotic epigenetic regulation has sex-specificity. In the *Drosophila* ovary, only one germ cell per germline cyst differentiates into oocyte while the rest enter into a cell cycle variation known as an endocycle to become nurse cells. Conversely, in the male germline, all mitotic germ cells enter meiosis and differentiate to sperm. Transdetermination from oocyte to nurse-like cells was observed when PcG components E(z) and Su(z)12 are knocked down in *Drosophila* female germline. This cell fate change results from derepression of Cyclin E and cyclin-dependent kinase inhibitor Dacapo upon loss of the repressive H3K27me3 mark (Iovino et al., 2013). However, such a cell fate switch does not occur in the male germline, suggesting that differences between female and male germline differentiation pathways require distinct epigenetic regulators.

Consistently, microRNAs, important post-transcriptional regulators, have distinct roles in regulating male versus female germline differentiation in *Drosophila*. Bam has critical, but distinct roles, in regulating male versus female germ cell differentiation (Fuller and Spradling, 2007). In meiotic male germ cells, microRNAs downregulate Bam protein but not *bam* mRNA through an interaction with the 3'UTR. Failure to achieve this downregulation leads to sperm differentiation defects (Eun et al., 2013). However, this regulation does not happen in the female germline. Another example includes male germline-specific regulation of the Wnt signaling pathway by microRNAs: both β-catenin and the downstream transcription factor TCF are downregulated by microRNAs in *Drosophila*. Loss of such an antagonization leads to male germline differentiation defects and decreased fertility (Pancratov et al., 2013). In addition, piRNAs are the largest group of small non-coding RNAs and play important roles in the germline. For example, a recent study using *Maelstrom* mutant mice lacking piRNAs uncovered increased retrotransposon LINE-1 activity and extensive elimination of oocytes, providing an interesting connection betweenpiRNA levels and fetal oocyte quality control (Malki et al., 2014). In *Maelstrom* mutant adult male mice, lack of piRNAs may indirectly lead to failure in the translation of many mRNAs required for sperm differentiation, resulting in male sterility (Castaneda et al., 2014).

During spermatid maturation, the switch from histones to protamines is a conserved process to ensure a highly compact paternal genome (reviewed by (Gaucher et al., 2010; Rathke et al., 2014)). The histone-to-protamine switch requires coordinated activity of histone variants and modified histones to destabilize nucleosome structure followed by histone degradation and replacement with small basic proteins such as

protamines. Both H4 hyperacetylation (Awe and Renkawitz-Pohl, 2010) and H3K79 methylation (Dottermusch-Heidel et al., 2014a; Dottermusch-Heidel et al., 2014b) are reported to be conserved modifications prior to this transition. Moreover, acetylation inhibition leads to little synthesized methyltransferase, suggesting that the methylation process depends on the acetylation. Replacement of histones with protamines is initiated by degrading core histones in an acetylation-dependent but polyubiquitin-independent manner in mice (Qian et al., 2013). Independent of histone removal, assembly of protamine-based chromatin in sperm requires the function of the canonical histone chaperone CAF1 (Doyen et al., 2013).

Transgenerational epigenetic inheritance

As discussed earlier in this review, histone modifying enzymes and modified histones are known for their important roles in regulating germline identity and activity. However, it is not well understood whether and how this information from parental germline could pass onto their progeny; and if so, whether somatic cells and/or germ cells of the progeny inherit this information. Recent published work sheds some light on this process. A recent study in *C. elegans* utilized elegant genetics to generate embryos that lack the H3K27me3-writing enzyme and can only inherit H3K27me3 from parents. Interestingly, under this condition H3K27me3 stays with the genome where the marker is generated for quite a few cell cycles in early embryos, suggesting that both sperm and oocyte can pass epigenetic information to early embryos. However, maintenance of histone modifications requires H3K27me3-writing enzymes, suggesting that modified histones and histone-modifying enzymes cooperate to pass the epigenetic information across generations (**Fig. 1-4**). A similar effect was also observed for another repressive

modification H3K9me2 acting with H3K9me2-writing enzyme (Gaydos et al., 2014). In addition to repressive marks, modifications associated with active transcription such as H3K4me2 can also be inherited through many generations. For example, in *C. elegans* the H3K4me2-demethylase is encoded by *spr-5*, whose mutants display decreased fertility across generations along with the global accumulation of H3K4me2 (Katz et al., 2009). This progressive sterility phenotype provides a sensitized background for screening for genetic modifiers, from which a cohort of histone modifying enzymes have been identified as either enhancers or suppressors, including H3K4me1/2-writing enzyme, H3K9me3-writing, reading and erasing enzymes (Greer et al., 2014) (**Fig. 1-4**). Because these enzymes have antagonizing functions toward either active (e.g. H3K4me1/2) or repressive (e.g. H3K9me3) marks, it suggests that a balance between euchromatin and heterochromatin is needed for transmitting epigenetic information properly to the offspring. Noticeably, a very recent study demonstrates a potential connection between transgenerational epigenetic inheritance and obesity in mice and human (Ost et al., 2014).

In vertebrates, DNA methylation is another important epigenetic player in addition to histone modifications. A recent study in zebrafish (**Fig. 1-5**) found that the paternal but not the maternal DNA methylation profiles are retained throughout early embryogenesis (Jiang et al., 2013). On the other hand, the maternal DNA methylome is reprogrammed and acts with the paternal DNA methylome to establish a totipotent zygotic epigenome (Potok et al., 2013). Because studies in mice and humans show a similar phenomenon (Hammoud et al., 2014; Hammoud et al., 2009), it is of particular interest to understand how gametogenesis resets chromatin while retaining certain

epigenetic information; and whether abnormalities in this process leads to transgenerationally inheritable diseases.

In addition to histone modifications and DNA methylation, piRNAs also contribute to transgenerational epigenetic inheritance. In *Drosophila*, maternally deposited piRNAs are important for transposon silencing in the next generation, without which progenies become sterile (Brennecke et al., 2008). Recent studies in multiple strains from two different *Drosophila* species demonstrate that transgenerationally transmitted piRNA can serve as a trigger to promote piRNA biogenesis by changing the chromatin state at piRNA precursor region in progenies (Le Thomas et al., 2014a; Le Thomas et al., 2014b) (**Fig. 1-5**). It has been shown that piRNAs and their associated proteins contribute to transgenerational gene silencing in germline in *C.elegans* (Ashe et al., 2012). More studies are needed to understand how different epigenetic mechanisms cooperate to ensure proper transgenerational inheritance to maintain somatic fitness as well as germline integrity and functionality.

FIGURES

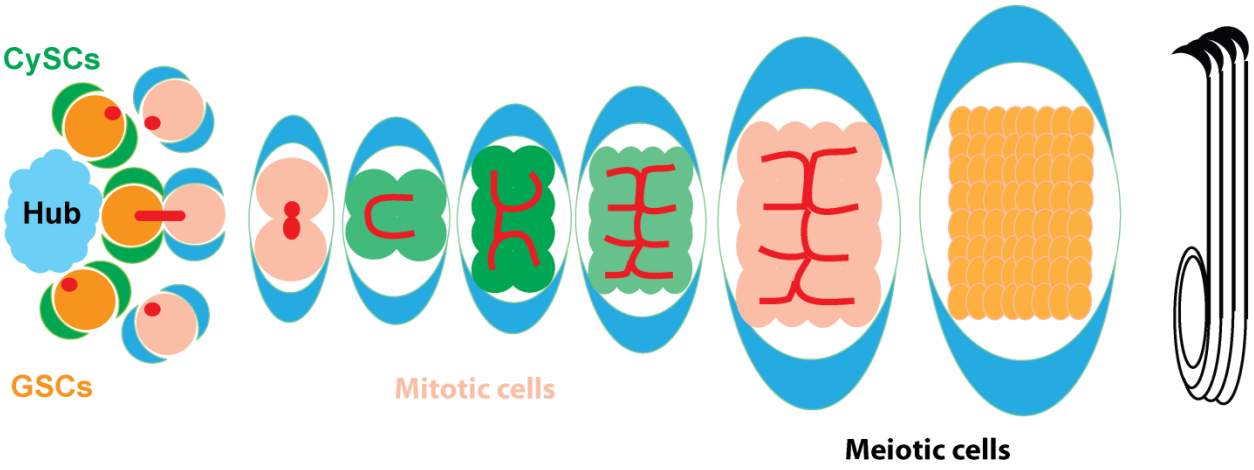


Figure 1-1: Drosophila spermatogenesis. GSCs divide to self-renew and produce spermatogonial cells, which undergo four mitotic divisions as amplification stage and transit into differentiating spermatocytes. Then spermatocytes differentiate to spermatid after two meiotic divisions, which finally mature to sperm.

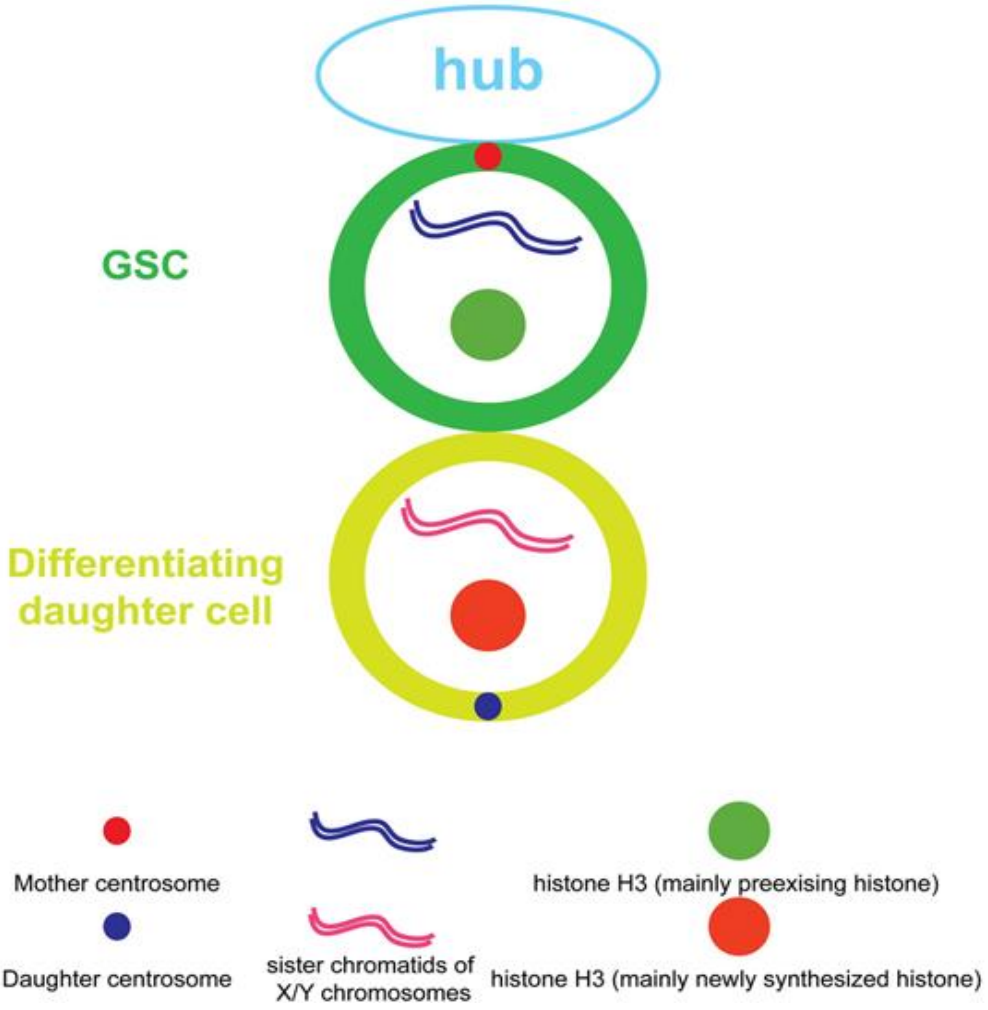


Figure 1-2: Non-random segregation of sister chromatids and histone H3 during asymmetric division of GSCs. Asymmetric GSC divisions give rise to two daughter cells: a self-renewed GSC (green) and a differentiating daughter cell (yellow). During this division, mother and daughter centrosomes with distinct microtubule nucleating capabilities are inherited asymmetrically; sister chromatids of X and Y chromosomes are segregated non-randomly, and preexisting H3 are preferentially segregated to GSC while newly synthesized H3 are mainly segregated to the other differentiating daughter cell. Potential crosstalk between non-random segregation of sex chromosome sister chromatids and preexisting *vs.* newly synthesized H3 might exist.

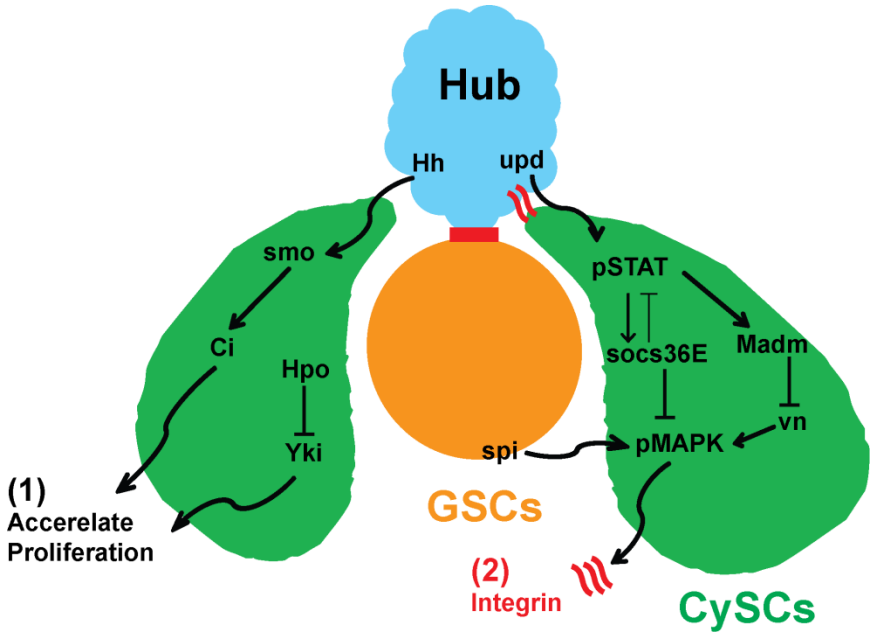


Figure 1-3: GSC and CySC competition in Drosophila testes. (1) Proliferative changes downstream of active Hh and Hpo signaling drive CySCs to outcompete neighboring GSCs. (2) MAPK pathways play crucial roles in CySCs competition through regulating Integrin levels. Lacking inhibition from socs36E or Madm leads to elevated pMARK in CySCs and causes out the competition of wild-type GSCs and CySCs. Both Socs36E expression and Madm localization are regulated by JAK-STAT signaling in CySCs.

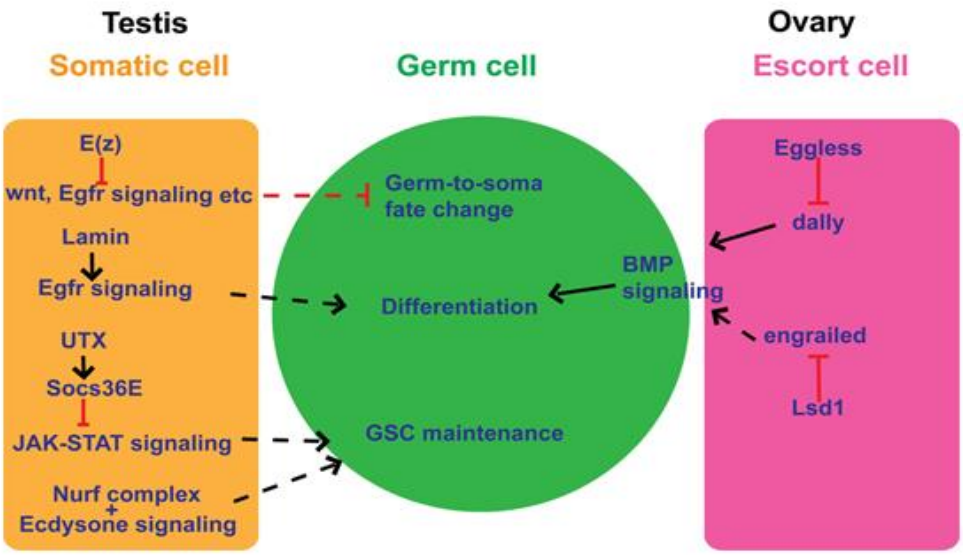


Figure 1-4: Non-cell-autonomous regulation of germline through somatic gonadal cells. In somatic cells of *Drosophila* testis, H3K27me3-methyltransferase E(z) might maintain germ cell fate through downstream signalings, such as Wnt or Egfr pathway(s). Egfr signaling can also be regulated by nuclear lamina to promote germ cell differentiation. The H3K27me3 demethylase dUTX can modulate JAK-STAT signaling through activating JAK-STAT signaling inhibitor Socs36E. In addition, chromatin remodeling complex component, such as Nurf301, cooperates with ecdysone signaling to maintain GSCs. In *Drosophila* ovary, BMP signaling directly represses transcription of the differentiation-promoting gene to maintain GSCs. In escort cells, H3K9me3-methyltransferase Eggless and H3K4me1/2-demethylase Lsd1 prevent ectopic BMP signaling outside the niche to regulate germline differentiation.

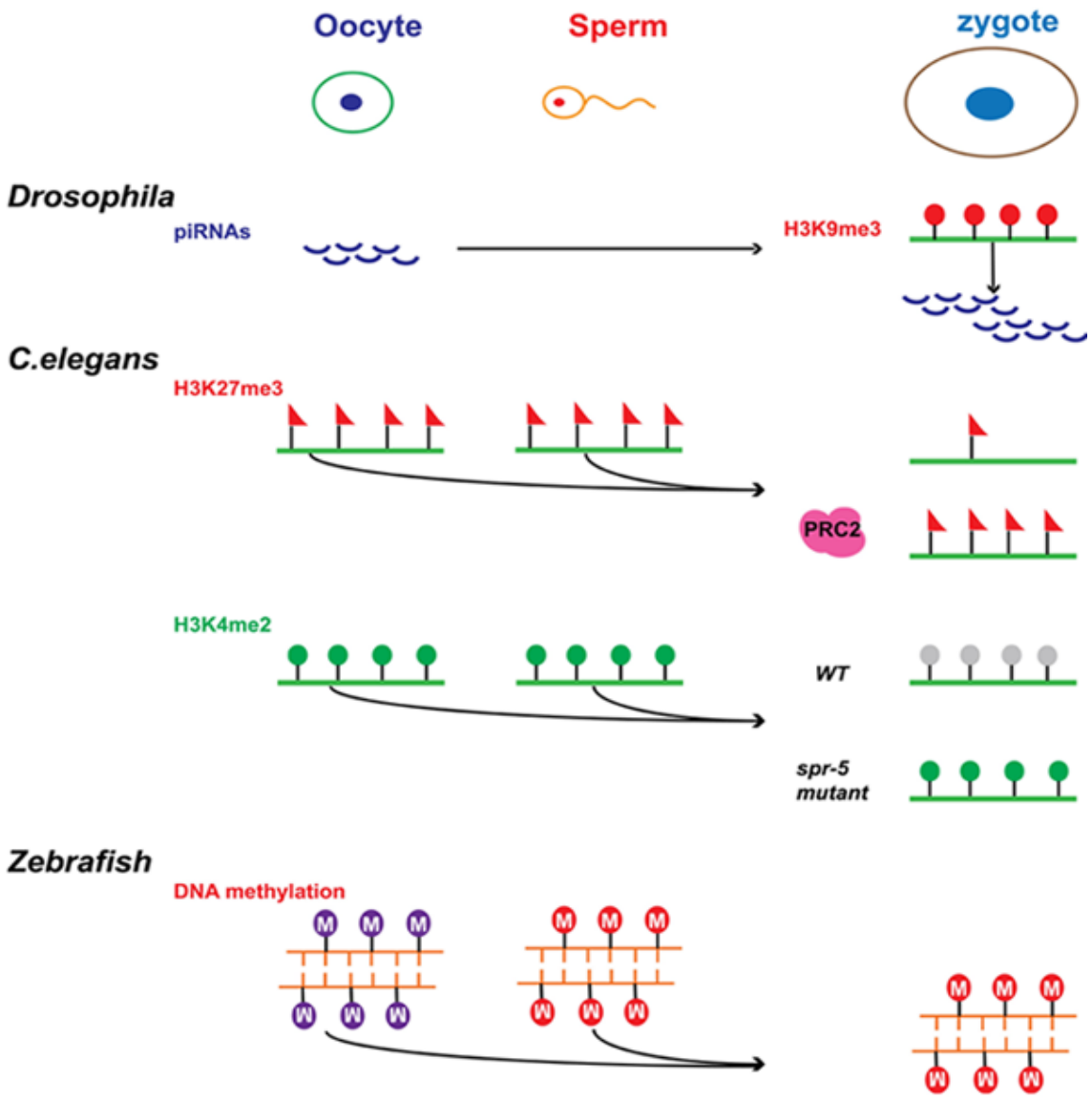


Figure 1-5: Transgenerational epigenetic inheritance in multiple organisms. In *Drosophila*, maternal piRNAs are transmitted to the next generation, whose function is important for transposon silencing in germline to maintain fertility. In addition, they also lead to H3K9me3 at piRNA clusters to promote piRNA biogenesis. In *C. elegans*, maternal and paternal PRC2-generated H3K27me3 can both be transmitted to embryos. Without the PRC2 enzyme, the H3K27me3 repressive mark only persists for several rounds of cell divisions. Different from H3K27me3, H3K4me2 needs to be erased by the

Spr5 demethylase in germ cells at each generation. Failure of erasure leads to heritable accumulation of H3K4me2 and misregulation of spermatogenesis gene expression in germ cells over 20 generations. Multiple chromatin factors, including H3K4me1/me2 and H3K9me3 methyltransferase, H3K9me3 demethylase, and H3K9me reader, can enhance or repress the sterility phenotype of *spr-5.* In *Zebrafish*, paternal DNA methylation pattern is maintained during embryo development, while the maternal DNA methylome undergoes reprogramming to resemble the paternal methylome.

Chapter 2

Enhancer of Polycomb represses transcription of Cyclin B during male germ cell differentiation

INTRODUCTION

Stem cell proliferation and differentiation are tightly associated with cell cycle machinery. For example, both mouse ESCs and human ESCs are characterized by a short G1 phase and a lengthening of G1 is found during differentiation (Becker et al., 2010; Calder et al., 2013; Coronado et al., 2013; Neganova et al., 2009). Interestingly, small molecule based manipulation of Cyclin D-CDK4/6 is sufficient to induce endoderm differentiation from hPSCs without exogenous signals, implying the tight interconnection between cell cycle and differentiation (Pauklin and Vallier, 2013). Such tight regulation from cell cycle machinery also applies to adult stem cells. Hematopoietic stem cells (HSCs) provide blood cells for a lifetime and thus their quiescence and proliferation require a balance between the two stations, which is linked to cell cycle regulation. Interestingly, fetal, adult, and aged HSCs show different cell cycle activities (Bowie et al., 2006; Catlin et al., 2011; Foudi et al., 2009; Kiel et al., 2007; Nygren et al., 2006; Wilson et al., 2009), suggesting complex regulatory mechanisms of HSC cell cycle (Pietras et al., 2011). In addition to the coordination with cell cycle regulators, another challenge stem cells face is how to protect genome-wide integrity during consecutive rounds of replication. For example, diminished DNA damage repair causes a progressive loss of HSCs during aging (Nijnik et al., 2007; Park and Gerson, 2005; Rossi et al., 2007). Defects in DNA damage detection and repair are found in diverse human diseases (Jackson and Bartek, 2009). Thus improved understanding of how DNA damage is treated at the molecular level in stem cell lineages will advance disease detection and management.

To decipher the molecular mechanisms of cell cycle regulation and DNA damage repair in a stem cell lineage, we focused on the genetically tractable model of germline stem cells (GSCs) in the Drosophila testes. Spermatogenesis initiates from the asymmetric cell division of

GSCs, localized at the apical tip of the testes. Gonialblast, the differentiated daughter cell of GSC, undergoes four rounds of mitosis, a transit amplifying spermatogonial stage. Then, 16 interconnected germ cells progress toward the pre-meiotic stage as primary spermatocytes, which enter a prolonged G2 phase, followed by two meiotic divisions (Fuller, 1998). Multiple cell cycle regulators have been identified to function during Drosophila germ cell proliferation. For example, String, the Drosophila homolog of cdc25, has a robust expression in GSCs and rapidly downregulate in differentiating daughter cells. Stem cell-enriched expression of String is essential for GSC maintenance (Inaba et al., 2011). While twine, another cdc25 homolog, is robustly expressed in primary spermatocyte and required for meiosis (Alphey et al., 1992; Courtot et al., 1992; White-Cooper et al., 1993). In addition, Cyclin B (Cyc B) is expressed in mitotic germ cells and downregulated in spermatocytes until the G2/M transition of the first meiosis. Distinctly, *Cyc B* RNA is detected in germ cells undergoing mitosis, negatively regulated upon completion of the spermatogonial stage and reinitiate expression in primary spermatocytes at prolonged G2 phase (White-Cooper et al., 1998). Transcriptional activation of *Cyc B* RNA in spermatocytes is mediated by meiotic arrest protein Aly. Moreover, translational repression of *Cyc B* RNA by RNA-binding protein Rpb4 and its binding partner Fest is found to downregulate Cyc B protein in spermatocytes undergoing a prolonged growing stage (Baker et al., 2015). A remaining question is how CycB transcription is repressed after four rounds of mitosis. It is also unknown if such a repression is a prerequisite for the transition from mitosis to meiosis. In addition, it remains unclear how DNA damage in germ cells accumulated after consecutive mitotic division are repaired.

Here, we characterize the function of chromatin factor E(Pc) in regulating CycB and DNA damage repair during germ cell differentiation. E(Pc) genetically interacts with Polycomb

(PcG) group and E(Pc) mutant functions as an enhancer of PcG phenotypes and also a suppressor of position effect variegation (Sato et al., 1983; Sinclair et al., 1998; Stankunas et al., 1998). These indicate E(Pc) might play a role in transcription and heterochromatin formation. Moreover, E(Pc) is identified as one component of Drosophila Tip60 (dTip60) chromatin-remodeling complex (Kusch et al., 2004). As a conserved complex from yeast to mammals, the Tip60 HAT complex is well studied and involved in both DNA damage response and transcriptional regulation (Cheng et al., 2008b; Gorrini et al., 2007; Kim et al., 2015; Lorbeck et al., 2011; Mattera et al., 2009; Murr et al., 2006; Squatrito et al., 2006; Sun et al., 2005). DNA DSBs can phosphorylate Drosophila H2Av, the only known H2A variant (van Daal et al., 1988), and its phosphorylation prevents radiation-induced apoptosis (Madigan et al., 2002). Phospho-H2Av is a substrate of dTip60 and acetylated at lysine 5. Acetylated phospho-H2Av are exchanged with unmodified H2Av at DNA lesions through chromatin remodeler Domino, an adenosine triphosphatase (Kusch et al., 2004). Besides DNA damage repair, Tip60-mediated acetylation of histone and transcription factors is well known to activate gene expression by loosening chromatin compaction to allow access of transcription machinery to DNA (Ehrenhofer-Murray, 2004). In contrast, repressive regulation of transcription has also been identified for Tip60 HAT in multiple organisms. For example, upon Tip60 KD in mouse ESCs, the majority of misregulated genes are upregulated. Direct binding of the Tip60 complex was found at promoters of 55% of the identified genes, arguing that Tip60 HAT also functions as a repressor in mESCs (Fazzio et al., 2008).

Here, focusing on one Tip60 component E(Pc), we demonstrated two catalytic activities of Tip60 HAT, histone acetylation and H2Av exchange, both function in the Drosophila male germline lineage. Reduced H4 acetylation occurs in germ cells without E(Pc) and thus leads to

gene misexpression, including excess cell cyclin CycB and reduced level of differentiation factor Bag-of-marble (Bam). With misexpressed genes, germ cells undergo extra mitotic divisions, instead of transiting to the spermatocyte stage. In addition to H4 acetylation, depletion of E(Pc) in germ cells also leads to accumulated phospho-H2Av, which induces a high percentage of dying germ cells. Similarly, germ cell defects are observed when HAT activity of Tip60 is abolished. In summary, E(Pc) is essential for functional HAT activity, which is critical for germline differentiation and maintenance.

RESULTS

E(Pc) is required intrinsically for germ cell maintenance and differentiation

Utilizing a transgenic line carrying a GFP-tagged genomic region of E(Pc), nuclear localization of E(Pc) GFP in GSCs, spermatogonial cells (SGs) and spermatocytes (SCs) were observed (**Fig. 2-1A-A'**). It is consistent with the previous finding that E(Pc) encodes a chromatin factor, which can bind to multiple sites on polytene chromosomes (Sinclair et al., 1998; Soto et al., 1995; Stankunas et al., 1998). To explore the intrinsic function of E(Pc) in germ cells, a *short hairpin* (*sh*) RNA targeting its coding region was driven via an early germ cell driver *Nanos-Gal4* (*Nos-Gal4*) (Van Doren et al., 1998) to generate *E(Pc)* knockdown (KD) flies. To validate the specificity of *E(Pc) shRNA*, E(Pc) GFP signal in germ cells was compared with neighboring wild-type cyst cells. Loss of E(Pc) protein specifically in the germ line lineage was observed (**Fig. 2-1B-B'**). We further examined potential germ cell defects through immunostaining with the germ cell marker Vasa. In *Nos-Gal4* control (*ctrl*) testes, the germ cell lineage initiates from the GSCs at the apical tip of the testes to SGs and SCs (**Fig. 2-1C**). In contrast, few Vasa-positive cells in 94% of *E(Pc)* KD testes (N=52) implied a severe loss of the germ cell lineage (**Fig. 2-1D**), suggesting E(Pc) is essential to maintain the germ cell lineage.

To explore potential germ cell defects before the loss, temperature-sensitive Gal80 expressed under the control of the tubulin promoter (Tub-Gal80^{ts}) (McGuire et al., 2003) was applied to temporal control *E(Pc) shRNA* expression. At permissive temperature (18°C), functional Gal80 is produced to inhibit Gal4 activity. While at the non-permissive temperature (29°C), without functional Gal80, Gal4 remains active and drives *E(Pc) shRNA* expression. At the permissive temperature, both *Tub-Gal80^{ts}, Nos-Gal4* (*ctrl*) and *Tub-Gal80^{ts}, Nos >E(Pc) shRNA* (*E(Pc)* KD) testes had GSCs surrounding hub, SGs, and SCs (**Fig. 2-2A, 2-2B**). By 5D

old after shifting to the non-permissive temperature, 55% of *E(Pc)* KD testes (N=20) showed spermatogonial overproliferation (**Fig. 2-2D**), giving rise to more than 16 germ cells within one cyst (white outline in **Fig. 2-2D**). Excess germ cells were not observed in *ctrl* testes at the same condition (**Fig. 2-2C**). This suggests that germ cells without E(Pc) proceed through more rounds of mitosis instead of differentiation. By 10D old after the temperature shift, 39% of *E(Pc)* KD testes (N=23) showed spermatogonial overproliferation (**Fig. 2-2F**), while 35% showed germ cell loss as shown previously (**Fig. 2-1D**). In contrast, 10D *ctrl* testes did not show these phenotype (**Fig. 2-2E**). In summary, E(Pc) is required intrinsically for germ cell maintenance and also to regulate the transition from proliferation to differentiation.

E(Pc) mutant germ cell clones were induced to further validate its function. Negatively labeled *E(Pc)* clones were generated through mitotic recombination using FLP/FRT system (Xu and Rubin, 1993) and $E(Pc)^{w3}$/+ allele (Boivin et al., 2003; Ji et al., 2012). After clonal induction (ACI), the percentage of testes with *E(Pc)* homozygous GSC clones were compared with *ctrl* testes at different time points (**Fig. 2-2K**). 2D ACI, 65% of testes had *E(Pc)* homozygous GSC clones, comparable with 77% for *ctrl* testes (**Fig. 2-2G, 2-2H, 2-2K**). 4D ACI, the percentage of testes with *E(Pc)* homozygous GSC clones dropped to 17%, while 69% *ctrl* testes retained GSC clones (**Fig. 2-2I, 2-2J, 2-2K**). 8D ACI, no testes with *E(Pc)* GSC clones was found, while 53% *ctrl* testes still retained GSC clones (**Fig. 2-2K**). GSC clonal analysis indicated E(Pc) is required cell autonomously for GSC maintenance.

2D and 4D ACI, the percentage of testes with GFP-negative *E(Pc)* homozygous SG clones were comparable with *ctrl* testes (**Fig. 2-2L**). Consistent with spermatogonial overproliferation induced by *E(Pc)* KD (**Fig. 2-2F**), 15% of *E(Pc)* SG clones (N=27) had more than 16 germ cells (yellow outline in **Fig. 2-2J-J'**) at 4D ACI. Interestingly, very few GFP

negative *E(Pc)* SC clones were found at different time points after induction (**Fig. 2-2H, 2-2J, 2-2M**). These results suggest germ cells without E(Pc) fail to differentiate into spermatocyte stage. Instead, they undergo extra mitotic divisions and are arrested at the proliferation stage. In addition, *E(Pc)* SG clones were not observed at 8D ACI (**Fig. 2-2L**), suggesting overpopulated *E(Pc)* SG clones found at 4D ACI possibly go through cell death.

Taken together, our KD and clonal analysis both support that E(Pc) functions cell autonomously to maintain the germline lineage and promote the transition from the mitotic proliferation stage to the meiotic differentiation stage.

E(Pc) in germ cells directly regulates CycB transcription

Overexpression of cell cycle regulator CycB was correlated with the extra mitotic division in the Drosophila female germline (Lilly et al., 2000). Consistent with its role to promote mitosis, low CycB expression in germ cells was found to block progress over the 2-cell spermatogonial stage in Drosophila testes (Monk et al., 2010). We postulated that upon loss of E(Pc), there might be increased CycB in germ cells, which would cause extra mitotic divisions. CycB staining in wild-type testes revealed positive signal in germ cells undergoing mitotic amplification at the apical tip of the testes (**Fig. 2-3A-A"**). Consistent with previous findings, CycB is downregulated upon completion of four rounds of mitosis, and not detectable in primary spermatocytes (Baker et al., 2015; White-Cooper et al., 1998). In contrast, in 66% of *E(Pc)* KD testes (N=21), CycB positive cells expanded from the apical tip (yellow dashed line in **Fig. 2-3B-B"**) and even resided distal from the hub (yellow outline in **Fig. 2-3B-B"**). This indicates that compromised E(Pc) might derepress CycB expression. This hypothesis was further validated through CycB staining in *E(Pc)* mutant clones. As described previously, GFP negative *E(Pc)* clones (N=5, **Fig. 2-3C**) are arrested in the transit amplifying stage, indicated by DAPI bright

nuclei (**Fig. 2-3C'**). These *E(Pc)* mutant germ cells are enriched for CycB (**Fig. 2-3C"**). To determine whether ectopic CycB induces or is a consequence of excess germ cells, loss of function allele $CycB^2$ was applied to reduce CycB level. The percentage of testes with spermatogonial tumors decreased from 43% of *E(Pc)* KD testes (N=28) to 5% of $CycB^2$/+, *E(Pc)* KD testes (N=40) (**Fig. 2-3D**). This rescue strongly suggests ectopic CycB is one causal reason of extra germ cell division.

To examine if ectopic CycB (**Fig. 2-3B"**) was caused by changes in mRNA levels, transcripts of *CycB* was measured through qRT-PCT. Invariably expressed gene RPL32 was used as an internal control. A significant 2-fold increase of *CycB* mRNA (p=0.02) was detected in *Tub-Gal80^{ts}, Nos >E(Pc) shRNA* (*E(Pc)* KD) testes, compared with *Tub-Gal80^{ts}, Nos-Gal4* (*ctrl*) testes (**Fig. 2-3E**). In addition, we explored if E(Pc) can directly bind to the *CycB* gene region by identifying E(Pc) direct binding targets in Drosophila S2 cells. Excitingly, CycB was identified as a direct target of E(Pc) based on enrichment of E(Pc) GFP in the *CycB* genomic region (**Fig. 2-3F**). Taken together, these results demonstrate E(Pc) can directly regulate *CycB* transcription to regulate germline development.

In summary, we found E(Pc) might downregulate CycB transcription in germ cells. In addition, an E(Pc)-mediated decrease of CycB is shown to be primarily important for the transition in male germ cells. It is consistent with the role of CycB role in mitotic divisions in the female germline (Lilly et al., 2000). This identifies an instrumental role of coordination between the cell cycle program and development stages for two distinct adult stem cell systems.

Depletion of E(Pc) in germ cells reduces H4 tetra-acetylation intrinsically

Similar to E(Pc)'s role in germ cell maintenance (**Fig. 2-1D, 2-2F, 2-2M**), yeast E(Pc) homolog *Epl1* mutant is essential for cell viability (Boudreault et al., 2003). The regulatory role

of Epl1 is mediated through modulating acetylation activity of Esa1, yeast homolog of Tip60 HAT (Boudreault et al., 2003). Thus, Epl1's function indicates that loss of E(Pc) might modulate H4 acetylation levels to regulate gene transcription. We firstly investigated if HAT activity is required to promote germ cell differentiation and maintain the germ cell lineage. The $dTip60^{E431Q}$ mutant was shown to be a dominant negative HAT-deficient form when it is ectopically expressed (Lorbeck et al., 2011). In *Nos> $dTip60^{E431Q}$* testes, ectopic $Tip60^{E431Q}$ in germ cells led to spermatogonial tumors in 33% of testes (**Fig. 2-4B**) (N=30) and severe germ cell loss (**Fig. 2-4C**) in 17% of testes (N=30). Consistently, germ cell phenotypes were significantly enhanced when E(Pc) levels were reduced with the allele $E(Pc)^1$ (**Fig. 2-4D**). This strongly suggests H4 acetylation mediated transcription might regulate male germ cell differentiation and maintenance. This prompted us to examine H4 acetylation level in germ cells without *E(Pc)*. Indeed, H4 tetra-acetylation (acetylation of the lysines at position 5, 8, 12 and 16) declined in *E(Pc)* GSC clones (N=24), compared with neighboring heterozygous GSCs (**Fig. 2-4E-E'"**, **2-4H**). A similar reduction of H4 tetra-acetylation also appeared in *E(Pc)* SG clones (**Fig. 2-4F-F'"**, **2-4H**) (N=33). Unchanged H4 level between *E(Pc)* GSC clones and neighboring heterozygous GSCs (**Fig. 2-4G-G"', 2-4H**) uncovered that decreased H4 tetra-acetylation is not caused by the reduction of the H4 level. The essential role of E(Pc) in genome-wide acetylation activity is consistent with yeast Epl1 (Boudreault et al., 2003), confirming the conserved role of the subunit within the HAT complex.

As an epigenetic marker normally associated with active transcription, H4 acetylation decrease suggests a dramatic change in transcription in *E(Pc)* germ cell clones. Epigenetic regulation has been elaborately characterized to influence germ cell differentiation (Feng and Chen, 2015). But it remains unclear if one modification can influence a second modification

exemplified by the synergistic coupling between H3 phosphorylation and acetylation (Cheung et al., 2000; Lo et al., 2000). Immunostaining with H3K4me3 (N=29), an epigenetic marker associated with transcriptionally active regions, and H3K27me3 (N=33), which normally label transcriptionally repressive regions, revealed that these epigenetic markers are not affected at the global level in *E(Pc)* GSC clones (**Fig. 2-5A-A", 2-5B-B", 2-5C**). This indicates loss of E(Pc) is not causing global changes at the transcription level.

E(Pc) is required for Bam expression in germ cells

Spermatogonial overpopulation in *E(Pc)* KD testes (**Fig. 2-2D**) or *E(Pc)* homozygous clones (**Fig. 2-2J**) phenocopies *Bam* loss of function phenotype. During spermatogenesis, differentiation factor Bam is detected in 4-16 spermatogonial cells. It is essential for the transition from the amplifying stage to the differentiated spermatocyte stage after reaching a threshold level (Gonczy et al., 1997; Insco et al., 2009; McKearin and Spradling, 1990). We hypothesized that compromised E(Pc) might reduce Bam expression. Immunostaining against Bam-C (cytoplasm Bam) was performed for *E(Pc)* KD testes to test Bam expression. In wild-type testes (N=18), Bam signal was not detected in GSCs, GB and 2 cell-stage germ cells (**Fig. 2-6A", red in Fig. 2-6A'"**), which are close to the hub. As previous found (Insco et al., 2009), Bam is detectable in 4-to-16 spermatogonia stage(**Fig. 2-6A", red in Fig. 2-6A'"**), which are the relatively small Vasa-positive cells (**Fig. 2-6A'**) and have bright DAPI fluorescence (**Fig. 2-6A**). In *E(Pc)* KD testes (N=31), germ cells localized at the apical tip of the testes express Bam (yellow dashed line in **Fig. 2-6B"**, red in **Fig. 2-6B'"**) as in wild-type testes. Moreover, 48% of *E(Pc)* KD testes (N=31) had excess germ cells forming away from the apical tip (yellow outline in **Fig. 2-6B-B"'**). These germ cells have intense DAPI fluorescence (yellow outline in **Fig. 2-6B**) and relatively small size based on Vasa (yellow outline in **Fig. 2-6B'**). Interestingly, these

overpopulated germ cells did not have detectable Bam (yellow outline in **Fig. 2-6B"**). Lack of Bam in these excess germ cells leads to the hypothesis that loss of the differentiation factor Bam results in germ cell overpopulation in *E(Pc)* KD testes.

Besides *E(Pc)* KD in all germ cells, Bam-C level in *E(Pc)* homozygous clones was compared with GFP-positive *E(Pc)* heterozygous cells. At the apical tip of the testes, Bam is expressed in 4-16 spermatogonial cells (yellow dashed line in **Fig. 2-6C"'**). These Bam positive germ cells have intense DAPI fluorescence (**Fig. 2-6C'**) and relative smaller cell size (**Fig. 2-6C"**), features of early germ cells. Remarkably, early germ cells with negative GFP (yellow outline in **Fig. 2-6C**), representing *E(Pc)* mutant clones (N=7), were found far away from the apical tip. Interestingly, none of these overproliferating germ cells express Bam. Lack of Bam expression in *E(Pc)* KD germ cells or *E(Pc)* clones support our hypothesis that E(Pc) is required for Bam expression. Finally, Bam was reintroduced to confirm the loss of Bam contributed to excess germ cell phenotype in *E(Pc)* KD testes. Testes from Bam-HA transgenic flies have 2.4-fold of Bam mRNA level, compared with wild-type testes (Eun et al., 2013). Consistent with our hypothesis, *Bam-HA, Tub-Gal80^{ts}, Nos >E(Pc) shRNA* testes (N=37) exhibit no excess spermatogonia (**Fig. 2-6D**), demonstrating that E(Pc) dependent Bam expression is crucial to specify four rounds of mitosis and transit to differentiation.

To examine if reduced Bam in *E(Pc)* mutant germ cells were caused by changes in mRNA levels, transcript of *bam* was measured through qRT-PCT. As described, RPL32 was used as an internal control. Different from a significant change of *CycB* transcripts (**Fig. 2-2E**), reduction of *bam* mRNA in *E(Pc)* KD testes is not significant, compared with *ctrl* testes (**Fig. 2-6E**). In addition, no enrichment of E(Pc) at *bam* genomic region (**Fig. 2-7**) in S2 cells, which further rules out direct regulation of bam expression. The previous study demonstrated post-

transcriptionally regulation of Bam mediated by microRNAs was found to contribute Bam downregulation in spermatocytes (Eun et al., 2013). Therefore, reduced Bam upon loss of E(Pc) in germ cells might rely on post-transcriptionally regulation.

GSCs without E(Pc) have normal JAK-STAT and BMP signaling activity

Overexpression of Bam and reduction of CycB rescue spermatogonial overproliferation phenotypes. Interestingly, germ cell loss is still observed in all Bam-Ha, *Nos>E(Pc) shRNA* testes (N=21) and *$CycB^2$/+, Nos>E(Pc) shRNA* testes (N=23). Therefore, different regulatory mechanisms appear to maintain the germline lineage.

There are three potential reasons for germ cell loss: impaired GSC cell division, premature differentiation, and cell death. EdU (5-ethynyl-2'-deoxyuridine) incorporation was performed to evaluate GSC division. No significant difference of EdU positive GSC percentage was found between *ctrl* (17%, N=13) and *E(Pc)* KD testes (21%, N=23). This suggested GSCs with compromised E(Pc) divide as often as wild-type GSCs.

To determine if premature differentiation causes GSC loss, expression of multiple GSC maintenance factors were compared between *E(Pc)* homozygous GSC clones and neighboring heterozygous GSCs. Unpaired (Upd) ligand from hub cells activates Janus kinase-signal transducer and activators of transcription (JAK-STAT) signaling in both GSCs and CySCs to maintain stem cell activity (Kiger et al., 2001; Leatherman and Dinardo, 2010; Tarayrah et al., 2015; Tulina and Matunis, 2001). Immunostaining of STAT92E, which reflect JAK-STAT signaling activity, showed enriched signal in GSCs and some gonialblast (**Fig. 2-8A-A"**) as previous described (Issigonis et al., 2009; Tarayrah et al., 2013). Analysis of *E(Pc)* GSC clone (N=14) and neighboring *ctrl* GSC revealed no difference of STAT92E (**Fig. 2-8A-A"**), implying that JAK-STAT signaling is not likely affected upon E(Pc) depletion. In addition to JAK-STAT

signaling, the BMP signaling pathway also regulates male GSC self-renewal (Kawase et al., 2004; Schulz et al., 2004; Shivdasani and Ingham, 2003). Similarly, pSMAD level, a readout of BMP signaling activity, was comparable between *E(Pc)* GSC clone (N=9) and *ctrl* GSC (**Fig. 2-8B-B"**), indicating that E(Pc) loss of function did not modulate BMP signaling. In addition to signaling regulation, hub-GSC interface adherens junctions, E-cadherin was shown to partially rescue GSC loss caused by STAT depletion (Leatherman and Dinardo, 2010). To address if delocalization of E-cadherin is induced in GSCs without E(Pc), immunostaining with E-cadherin was performed and its localization and level were not affected in *E(Pc)* GSC clones (N=22) (**Fig. 2-8C-C"**), implying that the interface junctions remained the same upon loss of E(Pc). Taken together, GSCs without E(Pc) have normal expression of stem cell maintenance factors.

Depletion of E(Pc) in germ cells causes accumulation of DNA double-strand breaks (DSBs) and severe cell death

After ruling out deficient GSC division and maintenance defects, potential germ cell death was further explored. A previous study found that the Tip60 HAT complex is responsible for exchanging phosphorylated H2Av (γH2Av) with unphosphorylated H2Av at DNA lesions (Kusch et al., 2004). Failure of exchange with unphosphorylated H2Av after DNA damage increased cell apoptosis (Ikura et al., 2000; Madigan et al., 2002). dTip60 mediated acetylation of histone H4 increases at sites of DNA damage and is required for efficient DNA repair (Ikura et al., 2000; Monk et al., 2010; Niida et al., 2010). Reduced H4 acetylation upon loss of E(Pc) (**Fig. 2-4E"', 2-4F"', 2-4H**) promoted us to hypothesize that germ cell loss might be caused by accumulated γH2Av and followed by cell death upon deletion of E(Pc).

Consistent with our hypothesis, *E(Pc)* homozygous GSC clones (N=15) have increased γH2Av signal (**Fig. 2-9A-A"'**, **2-9D**), compared with neighboring heterozygous GSCs. A similar

accumulation of γH2Av was detected in spermatogonial (SG) clones (N=31) (**Fig. 2-9B-B'", 2-9D**). γH2Av accumulation is not likely to be caused by increased H2Av level because H2Av was not significantly increased in *E(Pc)* clones (N=27) (**Fig. 2-9C-C"', 2-9D**). This evidence suggested that germ cells without E(Pc) failed to exchange γH2Av at DNA lesions and thus led to the accumulation of the DNA DSB marker.

To demonstrate cell death occurs after accumulating DNA DSBs, we performed lysotracker staining. Lysotracker accumulates in low pH cellular compartments and was shown to label dying spermatogonial cysts (Yacobi-Sharon et al., 2013). Previous studies found male germ cells undergo alternative cell death and most dying cells are recorded at the 4-16 spermatogonial stage (Yacobi-Sharon et al., 2013; Yang and Yamashita, 2015). In contrast, GSCs have a high potential to resist cell death in response to stimuli including irradiation and starvation, better than their progeny (Hasan et al., 2015). Lysotracker-positive spermatogonial cells were found in 51% of *E(Pc)* KD testes (N=63) (**Fig. 2-9F**), while 14% were found in *ctrl* testes (N=14) (**Fig. 2-9E**). Consistent with the significant cell death of spermatogonial zone, 89% of *E(Pc)* KD testes (N=45) don't have Bam positive germ cells (**Fig. 2-9H**), which represent the 4-16 spermatogonial cell stage in *ctrl* testes (**Fig. 2-9G**). It strongly suggests that E(Pc) is essential to prevent severe germ cell death intrinsically. Reminiscent to E(Pc)'s role in the male germline, female germlines with *E(Pc)* knockdown produce no eggs and undergo germ cell death (Yan et al., 2014). This indicates E(Pc)'s cell autonomous role to maintain the germline lineage is conserved among two adult stem cell systems.

DISCUSSIONS

Our study revealed that chromatin factor E(Pc) is required for Tip60 HAT activity in the male germline lineage. With compromised E(Pc), H2Av exchange with phospho-H2Av at DNA DSBs are affected and accumulation of phospho-H2Av is detrimental to germline cells (**Fig. 2-10**). In addition, upon E(Pc) deletion, global levels of H4 tetra-acetylation is reduced, which leads to ectopic CycB and reduced Bam expression. In wild-type germ cells, reduction of CycB and accumulation of Bam are crucial for the transition of germ cells from the proliferating stage to the differentiation stage. Thus, germ cells without E(Pc) fail to differentiate, instead, they undergo additional rounds of mitosis (**Fig. 2-10**). Take together, E(Pc) dependent acetylation is important to repair DNA DSBs to maintain the germline and also to regulate the expression of the cell cycle regulator CycB to govern the differentiation program.

Drosophila germ cell death is independent of p53.

Transmission of an intact genome between generations relies on proper germ cell development during gamete formation. During normal Drosophila spermatogenesis, one fifth of spermatogonial cysts undergoes spontaneous cell death. Being independent of canonical caspases, this alternative germ cell death is executed through lysosomes and mitochondrial protease (Yacobi-Sharon et al., 2013). Moreover, upon protein starvation, increased deaths are found for transit amplifying stage germ cells, which are essential to allow GSC survival in response to starvation (Yang and Yamashita, 2015). We found inefficient DNA DSB repair also lead to severe germ cell death to eliminate damaged genomes from passing on to next generation. Consistent with previous findings that Tip60 has transcription-independent roles in DNA damage responses (Squatrito et al., 2006), Tip60 HAT component E(Pc) cooperates with Tip60 in germ

cells to exchange the phospho-H2Av at DNA DSBs. Accumulated phospho-H2Av upon loss of E(Pc) induces severe germ cell death.

We further uncovered a potential regulator contributing to germ cell death in *E(Pc)*/*Tip60* KD germ cells. The ataxia telangiectasia mutant (ATM) protein kinase plays an important role to repair DNA damage through phosphorylation of downstream proteins (Shiloh and Ziv, 2013). Interestingly, activation of ATM upon DNA damage is mediated through Tip60 acetylation activity and loss of Tip60 is found to block ATM-dependent kinase activity in the human cell line (Sun et al., 2005). It is interesting to explore if ATM kinase activity is abolished in germ cells without E(Pc). As a main substrate of ATM, p53 activation and stability play a central role in regulating cellular transcriptome upon DNA damage stimulation (Banin et al., 1998; Canman et al., 1998; Rashi-Elkeles et al., 2011; Reinhardt and Schumacher, 2012; Siliciano et al., 1997). Moreover, C.elegans p53-like CEP-1 protein is a prominent factor mediating germ cell apoptosis induced by DNA damage (Derry et al., 2001; Schumacher et al., 2001). To explore if p53 is also involved in cell death in the Drosophila male germline, a dominant negative form of p53, p53R155H (Ollmann et al., 2000), was applied. 67% of spermatogonial cysts were lysotracker positive in *p53DN, Nos>E(Pc) shRNA* testes (N=39), compared with 57% in *Nos>E(Pc) shRNA* testes (N=21). This implied that Drosophila germ cell death is not mediated through p53, in contrast with its role in C. elegans germ cells. It is consistent with the conclusion that Drosophila germ cells undergo an alternative cell death pathway (Yacobi-Sharon et al., 2013).

Transcriptional downregulation of CycB is crucial for germ cell differentiation.

In Drosophila spermatogenesis, it is known that CycB expression in the meiotic stage is under the transcriptional activation of tTAF complex (White-Cooper et al., 1998) and translational repression of RNA binding protein Rbp4 and its binding partner Fest (Baker et al.,

2015). Our finding that H4 tetra acetylation-mediated transcriptional regulation of CycB provides another level of regulation of an important cell cycle regulator in the mitotic stage. More importantly, excess CycB is one reason of additional germ cell divisions and failure to differentiate. Consistent with our finding, ectopic CycB induced by overexpression of mRNA binding protein Held-out-wings (HOW) also contributes to cysts with 32 or 64 germ cells (Monk et al., 2010). In the Drosophila ovary, overexpression of CycB forces cysts to undergo an extra division and form 32 germ cell cysts (Lilly et al., 2000; Mathieu et al., 2013). The evidence confirms a critical role of CycB levels in balancing germ cell proliferation and differentiation in two adult stem cell systems.

Ectopic expression of CycB in germ cells with reduced H4 tetra-acetylation raises an interesting question, how does H4 acetylation negatively regulate transcription since acetylation is generally linked to transcriptional activation and associated with euchromatin. This is not only the case in Drosophila germ cells. Drosophila SL2 cells with *Tip60* KD have both genes upregulated and downregulated. And enrichment of Tip60 was found at promoters of a subset of these activated or repressed genes. This evidence strongly supports that H4 acetylation can also repress transcription, in addition to activation. More interestingly, changes of gene expression in SL2 cells treated with Trichostatin A (TCA), an inhibitor of HDAC, exhibit high overlap with Tip60 KD cells (Schirling et al., 2010). These findings suggest HAT and HDAC cooperate to regulate gene expression in Drosophila. Similar cooperation between Tip60 and HDAC are also found to mediate gene repression in human cells (Ai et al., 2007; Xiao et al., 2003) and mouse ESCs (Chen et al., 2013b; Fazzio et al., 2008). In addition to H4 acetylation, H3K56 acetylation is also thought to activate transcription through promoting nucleosome disassembly (Williams et al., 2008; Xu et al., 2005). A recent study discovered that H3K56 can reduce transcription of

newly replicated DNA in budding yeast (Voichek et al., 2016). Taken together, histone acetylation plays dual roles in transcriptional regulation. In addition, whether histone acetylation can function as an activator or repressor might be influenced by cooperation between Tip60 HAT and HDAC.

In summary, our findings identified that E(Pc) is required for Tip60 HAT activity in Drosophila male germ cells. Depletion of E(Pc) compromises exchange of phosphor H2Av at DNA DSBs and also derepresses CycB expression, which is harmful to tissue homeostasis. A repressive role of H4 acetylation identified here differs from previously established models of Tip60 HAT as a transcriptional activator. As an evolutionarily conserved complex (Squatrito et al., 2006), Tip60 HAT functions identified in germline cells will provide insight into its function in other organisms.

MATERIALS AND METHODS

Fly strains and husbandry

Fly stocks were raised under standard yeast/molasses medium at 25°C unless stated otherwise. The following flies were used: *E(Pc)^{1}* (Bloomington Drosophila Stock Center, BL3056), *E(Pc)^{w3}* (BL9396), *E(Pc) shRNA* (BL35271), *Domino shRNA* (BL41674), *dTip60E431Q* (from Felice Elefant, Drexel University, Philadelphia, Pennsylvania, USA), *Tj-Gal4* (Kyoto stock center, DGRC#104055), *Nos-Gal4/Cyo (Kiger et al., 2001)* , *Nos-Gal4/Cyo;Tub-Gal80^{ts}/TM6B* (from Y. Yamashita, University of Michigan, Ann Arbor, Michigan), *Bam-HA* (Eun et al., 2013) , *Stat92E^{06346}* (from N. Perrimon, Harvard Medical School, Boston, MA, USA), *Dom^{3}/Cyo*(BL9260), *CycB^{2}/Cyo* (BL6630), *p53^{R155H}*(BL8426).

To study E(Pc) and Tip60 function in germ cells, *E(Pc) shRNA* was crossed with *Nos-Gal4* at 25°C separately. To study intermediate germ cell phenotype induced by *E(Pc)* knocked down, flies with the following genotype were generated: *Nos-Gal4/Cyo;Tub-Gal80^{ts}/E(Pc)shRNA. And 1D old males were shifted to* 29°C to induce expression of *E(Pc)shRNA* by inactivating Tub-Gal80^{ts}.

To explore if the loss of CycB could rescue the spermatogonial overproliferation phenotype induced by *E(Pc)* knockdown in germ cells, following flies were generated: *Nos-Gal4/CycB^{2}; E(Pc)shRNA/Tub-Gal80^{ts}*, *Nos-Gal4/Cyo;Tub-Gal80^{ts}/E(Pc)shRNA.* 1D old males from 18°C were collected and shifted to 29°C. Immunostaining with Vasa was applied to study germ cell overproliferation phenotype.

To study if overexpressing E(Pc) in germ cells is sufficient to rescue germ cell loss phenotype, following flies were generated: *Nos-Gal4/E(Pc)cDNA; E(Pc)shRNA/+*, *Nos-Gal4/+*;

E(Pc)shRNA/+. Testes from 1D old males at 25°*C* were immunostained with Vasa to examine germ cell loss phenotype.

Clonal analysis

To generate *E(Pc)* clones, $E(Pc)^{1}$ and $E(Pc)^{w3}$ null alleles were recombined with *FRT42B* to generate *FRT42B,* $E(Pc)^{1}$*/Cyo* or *FRT42B,* $E(Pc)^{w3}$*/Cyo* flies. Adult flies with the following genotype: *hs-FLP; FRT42B, Ubi-GFP/ FRT42B,* $E(Pc)^{1}$ or *hs-FLP; FRT42B, Ubi-GFP/ FRT42B,* $E(Pc)^{w3}$ and control flies *hs-FLP; FRT42B, Ubi-GFP/ FRT42B* were raised at 25 °C until pupal stage. Then they were heat-shocked for 2 hours each on two consecutive days. Adult flies 1D after heat shock were collected and aged until dissection.

Immunofluorescence

Testes were dissected and immunostained as previous described (Eun et al., 2014). Primaries antibodies *are:* Vasa (rabbit, 1:200, Santa Cruz, sc-30210), Vasa (rat, 1:50, DSHB), Zfh-1 (Rabbit, 1:5000, from R. Lehmann), Fas III (Mouse, 1:100, DSHB, 7G10), Armadillo (Mouse, 1:200, DSHB, N2 7A1), α-spectrin (Mouse, 1:50, DSHB, 3A9), Eya (mouse, 1:25, DSHB, 10H6), GFP (chicken, 1:1000, Abcam, ab13970), STAT92E (rabbit, 1:800, from Denise Montell, UC Santa Barbara), pSMAD (rabbit, 1:100, Abcam, ab52903), DE-Cad (Rat, 1:20, DSHB, DCAD2), H3K27me3 (rabbit, 1:2000, Millipore, #07-449), H3K4me3 (rabbit, 1:2000, Millipore, #07-473), H2Av (rabbit, 1:500, Active motif, 39716), γ-H2Av (rabbit, 1:1000, Rockland), H4 Pan acetylation (rabbit, 1:500, from Keji Zhao, NHLBI, NIH), Bam-C (mouse, 1:20, DSHB, bam), CycB (mouse, 1:20, DSHB, F2F4). For CycB and Bam staining, testes were dissected in 1X PBS and transferred onto slides. Slides were frozen in liquid nitrogen, followed by fixation in chilled methanol for 5 mins and acetone for 2 mins. Then testes were incubated

with primary antibodies as mentioned.

Secondary antibodies were all Alexa Fluor series (1:200, Molecular Probes). Images were taken with Zeiss LSM 510 META or LSM 700 using LSM software. Images were processed using Adobe Photoshop. Lysotracker (Invitrogen, L-7528) was used to label cells undergo cell death, following the manufacturer's protocol. Edu incorporation was performed with Click-iT Edu Alexa Fluro 488 imaging kit (Invitrogen C10083). Dissected testes were incubated with Edu solution for 30min, followed by fixation and immunostaining steps described.

Chromatin Immunoprecipitation (ChIP)

To perform S2 cell ChIP, transfection with 1ug Actin-Gal4 and 1ug UASp-E(Pc) cDNA-GFP plasmids were done following manuscript of Effectene® Transfection Reagent (Qiagen). S2 cells were split the day before the transfection experiment. On the day of transfection, seed 4X10^6 cells per 25 cm2 flask in 4ml growth medium containing serum and antibiotics. After transfection, S2 cells were incubated at RT for 40-48h to obtain maximal levels of gene expression. S2 cell fixation and following ChIP were performed with ChIP-IT high sensitivity kit (Active motif) following the manufacturer's instructions. Sonication of S2 cells was done with Bioruptor sonicator using following setting 0.5min on, 1min off for total 15min. Fragment size was around 500-600bp. ChIP grade GFP antibody (Abcam, ab290) were used to pulled down sheared chromatin. Two independent biological replicates were performed for both ChIP experiment.

Quantitative RT-PCR

Testes cDNA preparation and RT-PCR were performed and analyzed as previous described (Chen et al., 2011). Each PCR reaction was performed in triplicates and Ct values were averaged. And three biological replicates were used.

Primers:

Bam forward: 5'-ACTCAGCGCATGGAGAGATTGCTA-3'

Bam reverse: 5'-AGTAGCGGTGCTCCAGATCCATTT-3'

CycB forward: 5'-CTGTTCGTTTCGTGTTCGTTAAA -3'

CycB reverse: 5'-CAAGGGACTCCAGCAGATTAC -3'

Rpl32 forward:5'-CATGCTGCCCACCGGATTCAAGAAG-3'

Rpl32 reverse: 5'-CTCGTTCTCTTGAGAACGCAGGCGA-3'

Intensity Quantification

Values of histone modifications were quantified using Image J software: Nucleus was determined by DAPI signal and raw reading from GFP-positive cells were compared with neighboring GFP-negative cells within the same testes.

FIGURES

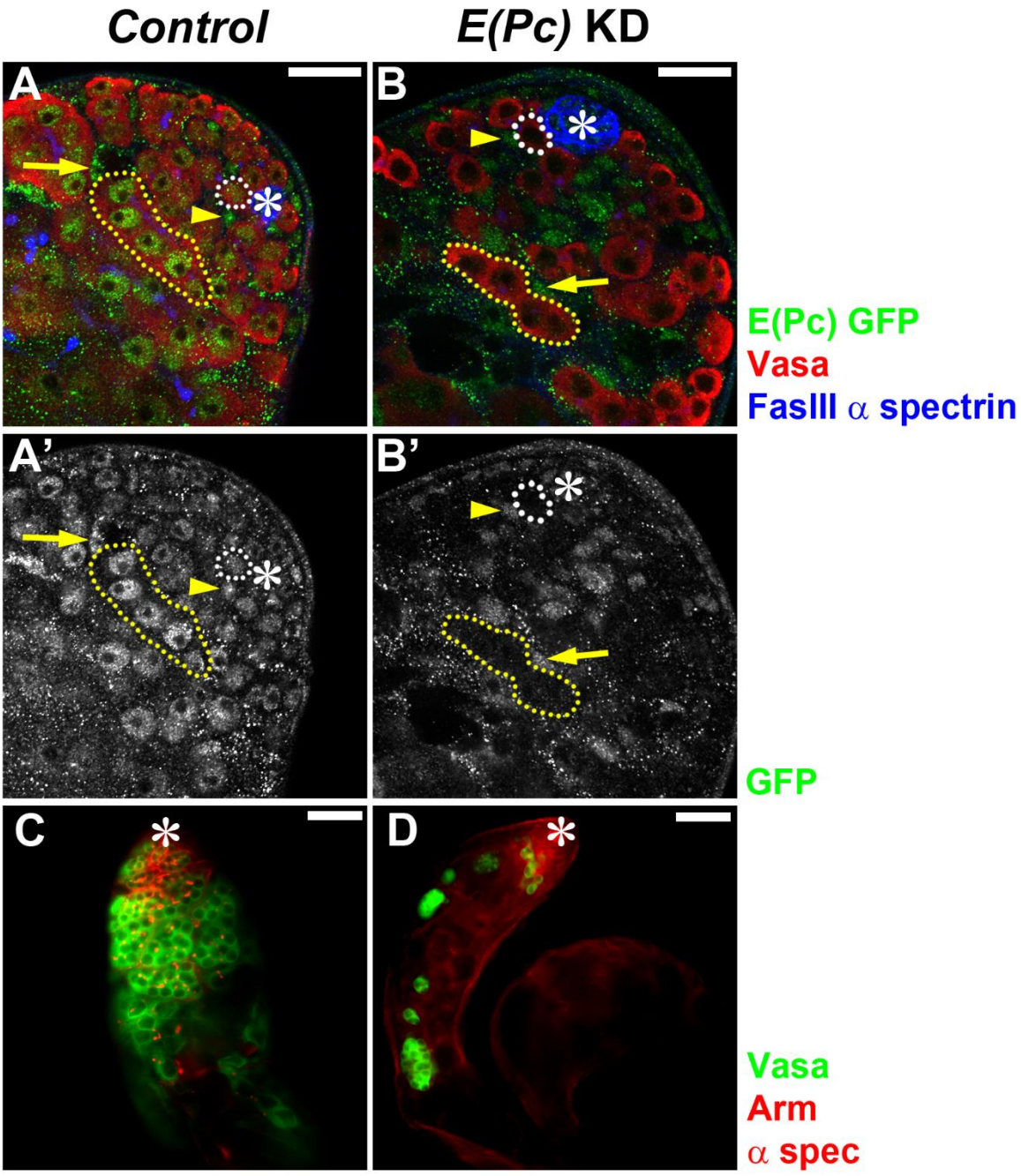


Figure 2-1: *E(Pc) shRNA* is reliable to specifically knockdown E(Pc).

(**A-B**) *Control* (*Ctrl, E(Pc)-GFP, Nos-Gal4*) and *E(Pc)* KD (*E(Pc)-GFP, Nos>E(Pc) shRNA*) testes stained with the germ cell marker Vasa, hub marker FasIII and fusome marker α spectrin.

GFP was tagged to *E(Pc)* genomic region. GSCs (white outline) and CySCs (yellow arrowhead) are close to the hub (asterisk). Spermatogonia (yellow outline) and accompanying cyst cells (yellow arrow) are several cell diameters away from the hub. In *ctrl* testes, E(Pc) GFP is expressed in both GSCs (white outline in **A'**), spermatogonia (yellow outline in **A'**), CySCs (yellow arrowhead in **A'**) as well as cyst cells (yellow arrow in **A'**). In *E(Pc)* KD testes, E(Pc) GFP is almost undetectable in both GSCs (white outline in **B'**) and spermatogonia (yellow outline in **B'**), while expression in CySCs (yellow arrowhead in **B'**) and cyst cells (yellow arrow in **B'**) are not affected. Asterisk: Hub. Scale Bar: 20 μm. **(C-D)** *Ctrl* (*Nos-Gal4*) and *E(Pc)* KD (*Nos>E(Pc) shRNA*) testes immunostained with the germ cell marker Vasa, somatic cells marker Arm, and fusome marker α spectrin. Compared with *ctrl* (*Nos-Gal4*) testes (**C**), severe germ cell loss was found in *E(Pc)* KD testes (**D**). Asterisk: Hub. Scale Bar: 50 μm.

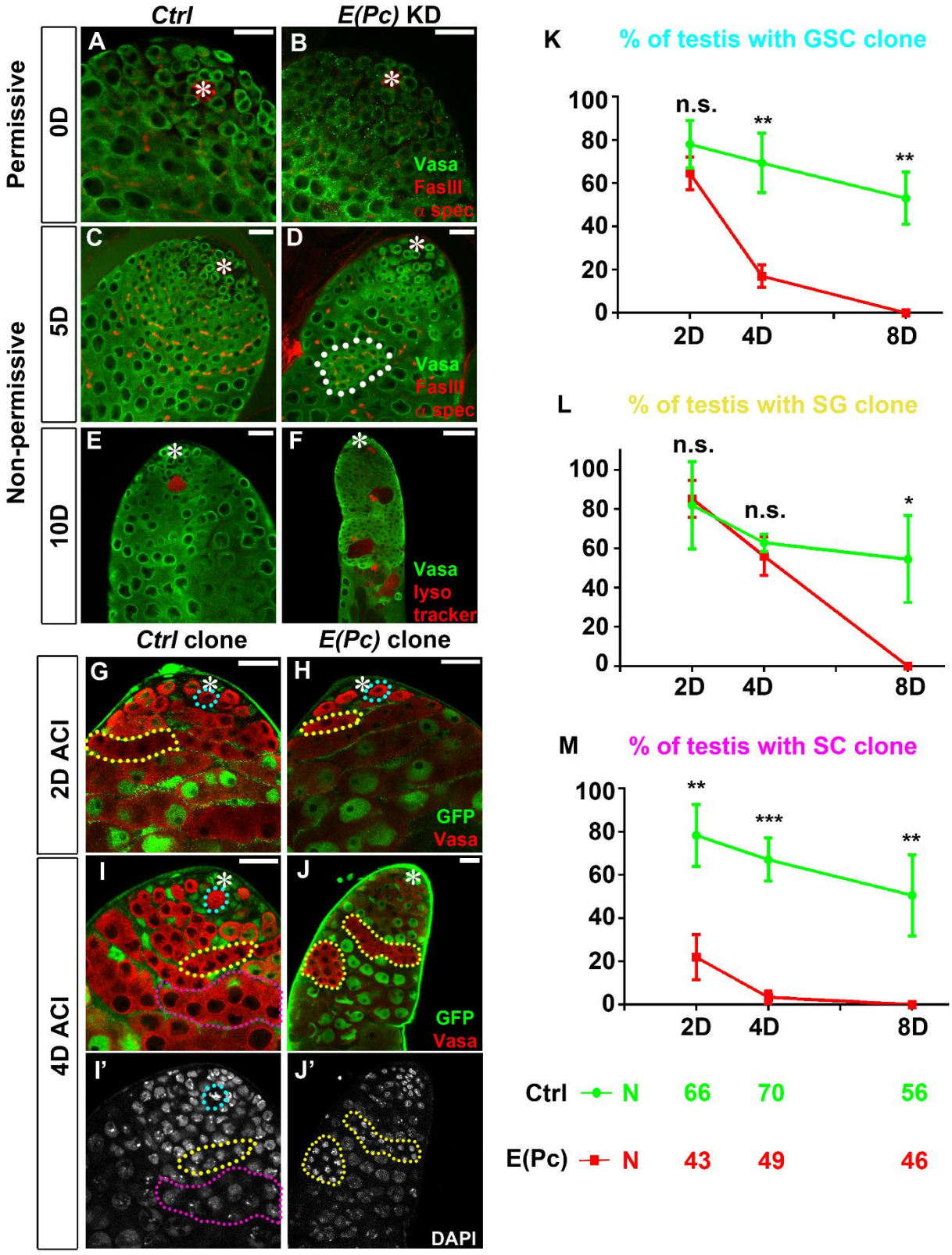
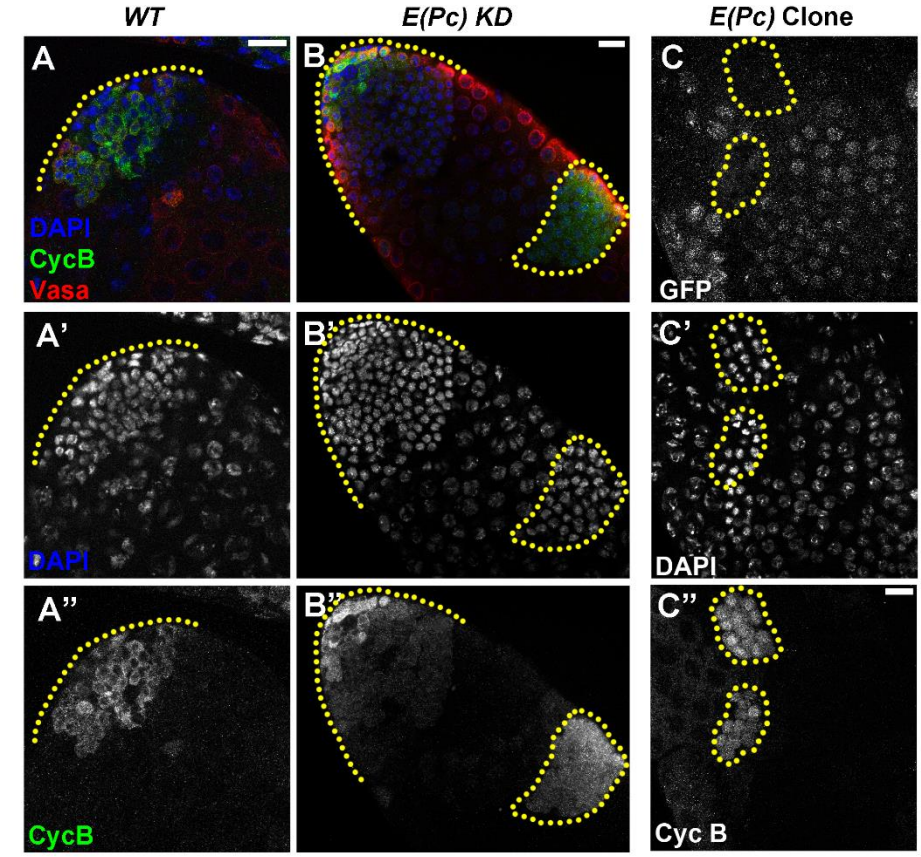
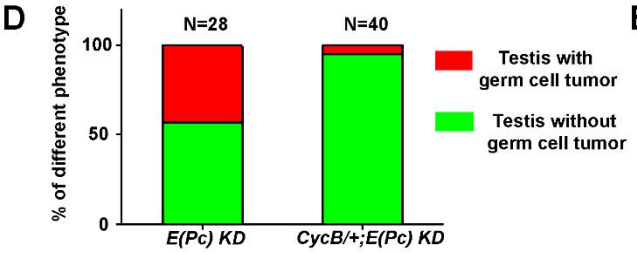
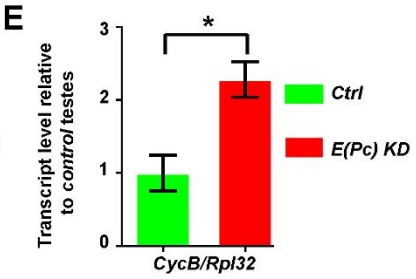

Ctrl
E(Pc) KD
Permissive
Non-permissive
0D
5D
10D
A
B
C
D
E
F
Vasa
FasIII
α spec
Vasa
FasIII
α spec
Vasa
lyso
tracker
Ctrl clone
E(Pc) clone
2D ACI
4D ACI
G
H
I
J
I'
J'
GFP
Vasa
GFP
Vasa
DAPI
K
% of testis with GSC clone
100
80
60
40
20
0
n.s.
**
**
2D
4D
8D
L
% of testis with SG clone
n.s.
n.s.
*
M
% of testis with SC clone
**
***
**
Ctrl N 66 70 56
E(Pc) N 43 49 46

Figure 2-2: E(Pc) is required for GSC maintenance and germ cell transition from the transit amplification stage to differentiation intrinsically.

Tub-Gal80^{ts}, Nos-Gal4 control (*ctrl*) and *Tub-Gal80^{ts}, Nos> E(Pc) shRNA* (*E(Pc)* KD) testes at permissive temperature (18°C) (**A-B**) and non-permissive temperature (29°C) (**C-F**) are immunostained with Vasa to stain the germ cells, FasIII to show the hub (asterisk) and α spec to show fusomes. At the permissive temperature, germ cells in *E(Pc)* KD testes (**B**) show no difference from *ctrl* testes (**A**). However, *E(Pc)* KD testes at 5D after temperature shift (**D**) have more than 16 germ cells within one cyst (white outline), which is not observed in *ctrl* testes at same condition (**C**). 10D after the temperature shift, *ctrl* testes show mild germ cell death based on lysotracker staining (**E**). (**F**) In contrast, multiple lysotracker positive dying cysts are observed in *E(Pc)* KD testes. (**G-J"**) GFP is used to identify *ctrl* and *E(Pc)* null clones. (**G-H**) 2D after clonal induction (ACI), GFP negative *ctrl* GSC clone (cyan outline in **G**) and *E(Pc)* null GSC clone (cyan outline in **H**), and GFP-negative *ctrl* spermatogonial (SG) clone (yellow outline in **G**) as well as *E(Pc)* null SG clone (yellow outline in **H**) can be observed. (**I-I'**) 4D ACI, *ctrl* GSC clone (cyan outline), SG clone (yellow outline) and spermatocyte (SC) clone (magenta outline) are observed. (**J-J'**) 4D ACI, *E(Pc)* null SG clone with bright DAPI intensity (**J'**) have more than 16 germ cells with one cyst (yellow outline, 64 germ cells). No GSC clone and SC clone are found for testes shown. (**K-M**) Quantification of testes with GSC clone (**K**), SG (**L**), SC (**M**) clones. Data shows average ± SD. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$. Asterisk: Hub. Scale Bar: 20 μm.


WT
E(Pc) KD
E(Pc) Clone
A
B
C
DAPI
CycB
Vasa
GFP
A'
B'
C'
DAPI
DAPI
A''
B''
C''
CycB
Cyc B


D
% of different phenotype
100
50
0
N=28
N=40
Testis with germ cell tumor
Testis without germ cell tumor
E(Pc) KD
CycB/+;E(Pc) KD


E
Transcript level relative to control testes
3
2
1
0
*
Ctrl
E(Pc) KD
CycB/Rpl32

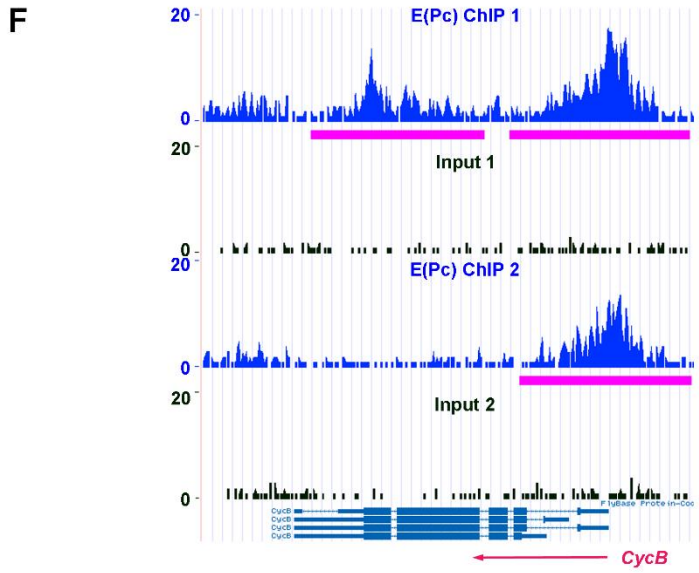
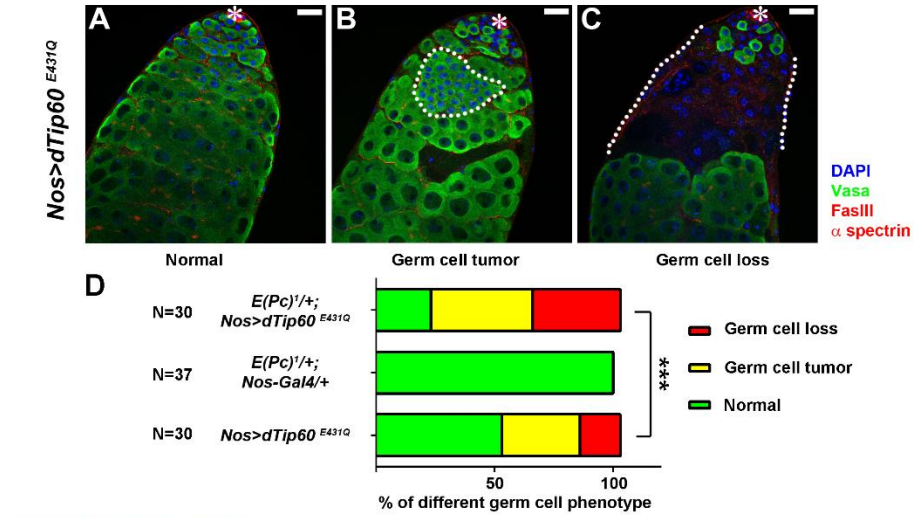
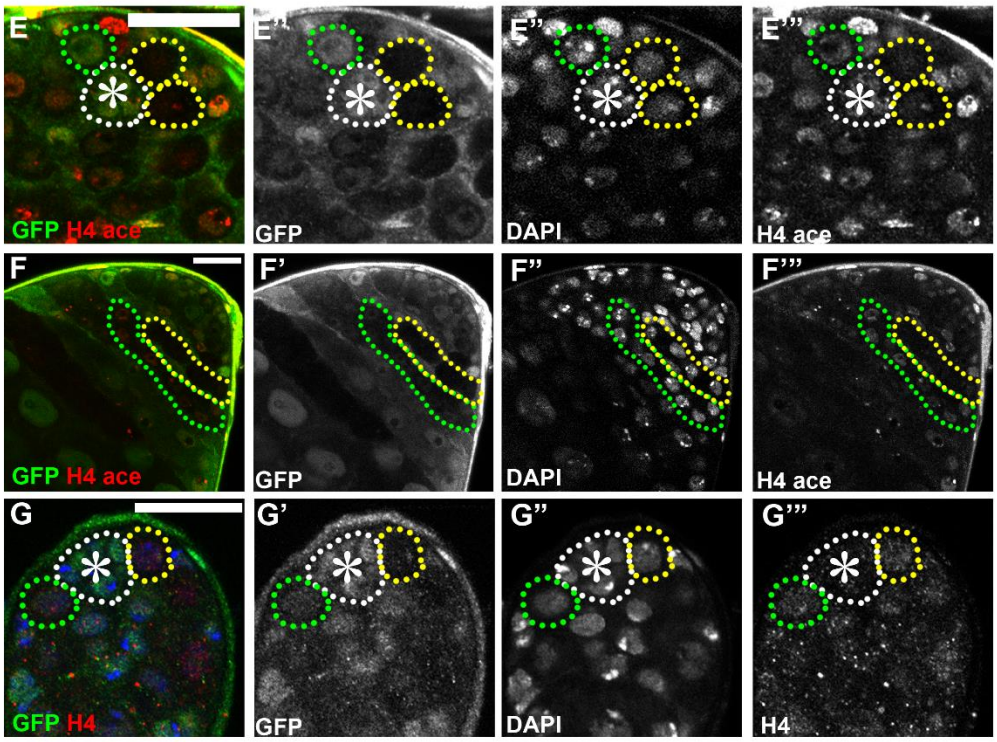

F
E(Pc) ChIP 1
Input 1
E(Pc) ChIP 2
Input 2
20
0
CycB

Figure 2-3: E(Pc) in germ cells is required to downregulate transcription of Cyclin B.

(**A-A"**) In *wild-type* (*WT*) testes, CycB positive (green in **A**, **A"**) cells have DAPI bright nuclei (blue in **A**, **A'**) and they localize at the apical tip of testes (yellow dashed line). (**B-B"**) In *Tub-Gal80^{ts}, Nos>E(Pc) shRNA* (*E(Pc)* KD) testes, CycB positive (green in **B**, **B"**) and Vasa-positive (red in **B**) germ cells at the apical tip (yellow dashed line) expand, compared with WT testes. In addition, CycB positive cysts (yellow outline) are found far away from the apical tip of the testes. (**C-C"**) GFP negative *E(Pc)* null (yellow outline) clones have relative high CycB expression. Scale Bar: 20 μm. (**D**) Percentage of testes with excess germ cells. (**E**) Quantitative RT-PCR of *CycB* mRNA in *ctrl* (*Tub-Gal80^{ts}, Nos-Gal4*) and *E(Pc)* KD (*Tub-Gal80^{ts}, Nos>E(Pc) shRNA*) testes. Data shows mean ± SEM (standard error of the mean) based on three independent biological replicates. Transcript level in *ctrl* testes is set to 1. p=0.02. (**F**) A genome browser snapshot of the *CycB* gene region. Compared with the input control, E(Pc) GFP shows enrichment at the genomic region of *CycB* in S2 cells. Peaks are indicated by magenta lines. Two independent replicates are shown here.


Nos>dTip60 E431Q
A
B
C
DAPI
Vasa
FasIII
α spectrin
Normal
Germ cell tumor
Germ cell loss
D
N=30
E(Pc)1/+; Nos>dTip60 E431Q
N=37
E(Pc)1/+; Nos-Gal4/+
N=30
Nos>dTip60 E431Q
***
Germ cell loss
Germ cell tumor
Normal
50
100
% of different germ cell phenotype


E
E'
E''
E'''
GFP H4 ace
GFP
DAPI
H4 ace
F
F'
F''
F'''
GFP H4 ace
GFP
DAPI
H4 ace
G
G'
G''
G'''
GFP H4
GFP
DAPI
H4

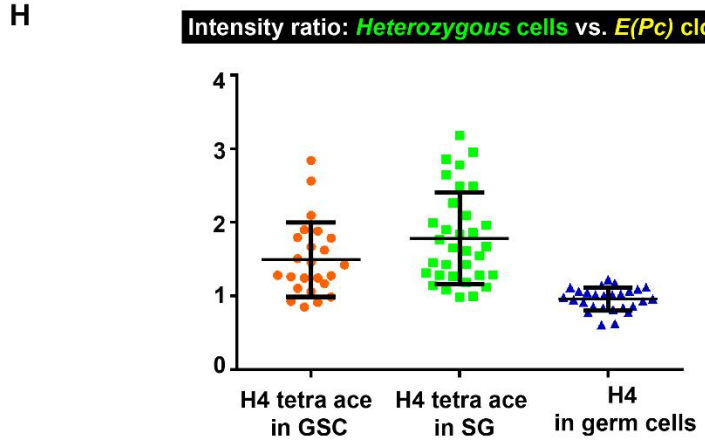

H
Intensity ratio: Heterozygous cells vs. E(Pc) clone
4
3
2
1
0
H4 tetra ace in GSC
H4 tetra ace in SG
H4 in germ cells

Figure 2-4: E(Pc) cooperates with histone acetyltransferase Tip60 to regulate germ cell differentiation through modifying acetylation levels.

(**A-C**) *Tip60*^{E431Q} is a HAT-deficient dominant negative mutation. *Nos>dTip60*^{E431Q} testes stained with nuclei marker DAPI, the germ cell marker Vasa, hub marker FasIII and the fusome marker α spectrin. They exhibit normal morphology (**A**), spermatogonial overproliferation (white outline in **B**), and germ cell loss (white dashed line in **C**) phenotype at different levels. Hub: asterisk. Scale Bar: 20 μm. (**D**) Quantification of germ cell phenotype. Heterozygous *E(Pc)*^{1}/+ enhances germ cell phenotype in *Nos>dTip60*^{E431Q} testes. ***p<0.005. (**E-G”’**) Apical tip of testes immunostained with GFP (**E’, F’ and G’**) to identify GFP negative *E(Pc)* null clones and DAPI (**E”, F” and G”**) to show nuclei. Immunostaining with H4 tetra-acetylation (**E’”**) reveals reduced H4 tetra-acetylation in *E(Pc)* GSC clones (yellow outline in **E”’**), compared with neighboring heterozygous GSC clones (green outline in **E”’**). Decreased H4 tetra-acetylation is also detected in *E(Pc)* SG clones (yellow outline in **F”’**), compared with neighboring heterozygous SG clones (green outline in **F”’**). No difference of H4 level is detected between *E(Pc)* GSC clones (yellow outline in **G”’**) and heterozygous GSC clones (green outline in **G”’**). Asterisk: Hub. Scale Bar: 20 μm. (**H**) Quantification of the intensity of H4 tetra-acetylation and H4 signal between heterozygous and *E(Pc)* germ cell clones. GSCs: H4 ace^{ctrl}/H4 ace^{clone}=1.49 ± 0.49. SG: H4 ace^{ctrl}/H4 ace^{clone}=1.78 ± 0.61. Germ cells: H4^{ctrl}/H4^{clone}=1.00 ± 0.17. Data is shown as mean ± SD.

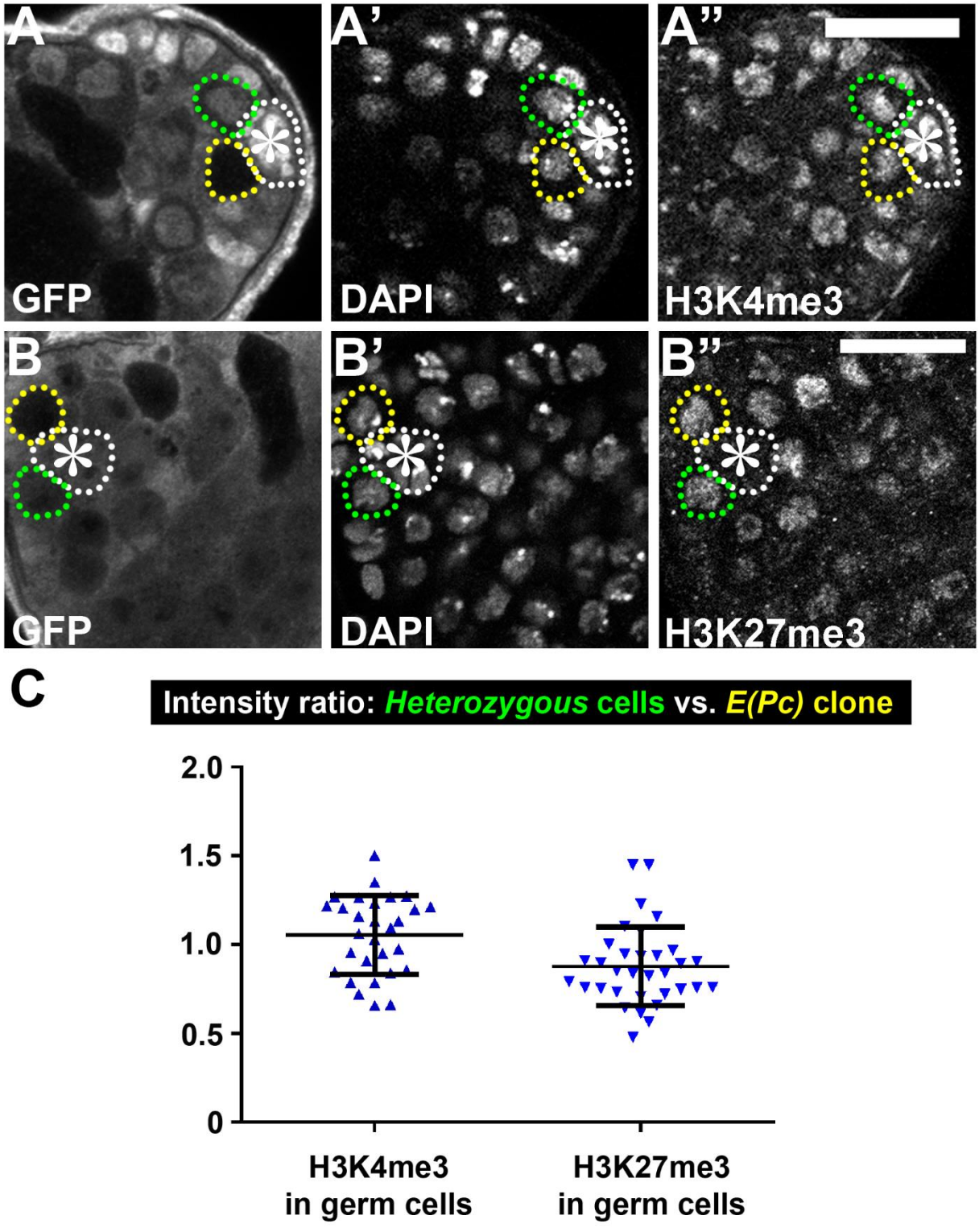


Figure 2-5: Level of H3K4me3 and H3K27me3 are not changed in *E(Pc)* null GSC clones.

(**A-B”**) GFP negative *E(Pc)* null clones (yellow outline) are compared with *E(Pc)* heterozygous clones (green outline). DAPI (**A’** and **B’**) is stained to identify nuclei. H3K4me3 (**A”**) and H3K27me3 (**B”**) level are not changed in *E(Pc)* null clone compared with neighboring *E(Pc)* heterozygous clone. Hub: Asterisk and white outline. Scale Bar: 20 μm. (**C**) Quantification of the intensity of H3K4me3 and H3K27me3 signal between heterozygous germ cells and *E(Pc)* null germ cell clones. Germ cells: $H3K4me3^{ctrl}$/ $H3K4me3^{clone}$=1.05 ± 0.22. Germ cells: $H3K27me3^{ctrl}$/ $H3K27me3^{clone}$=0.88 ± 0.22. Data is shown as mean ± SD.

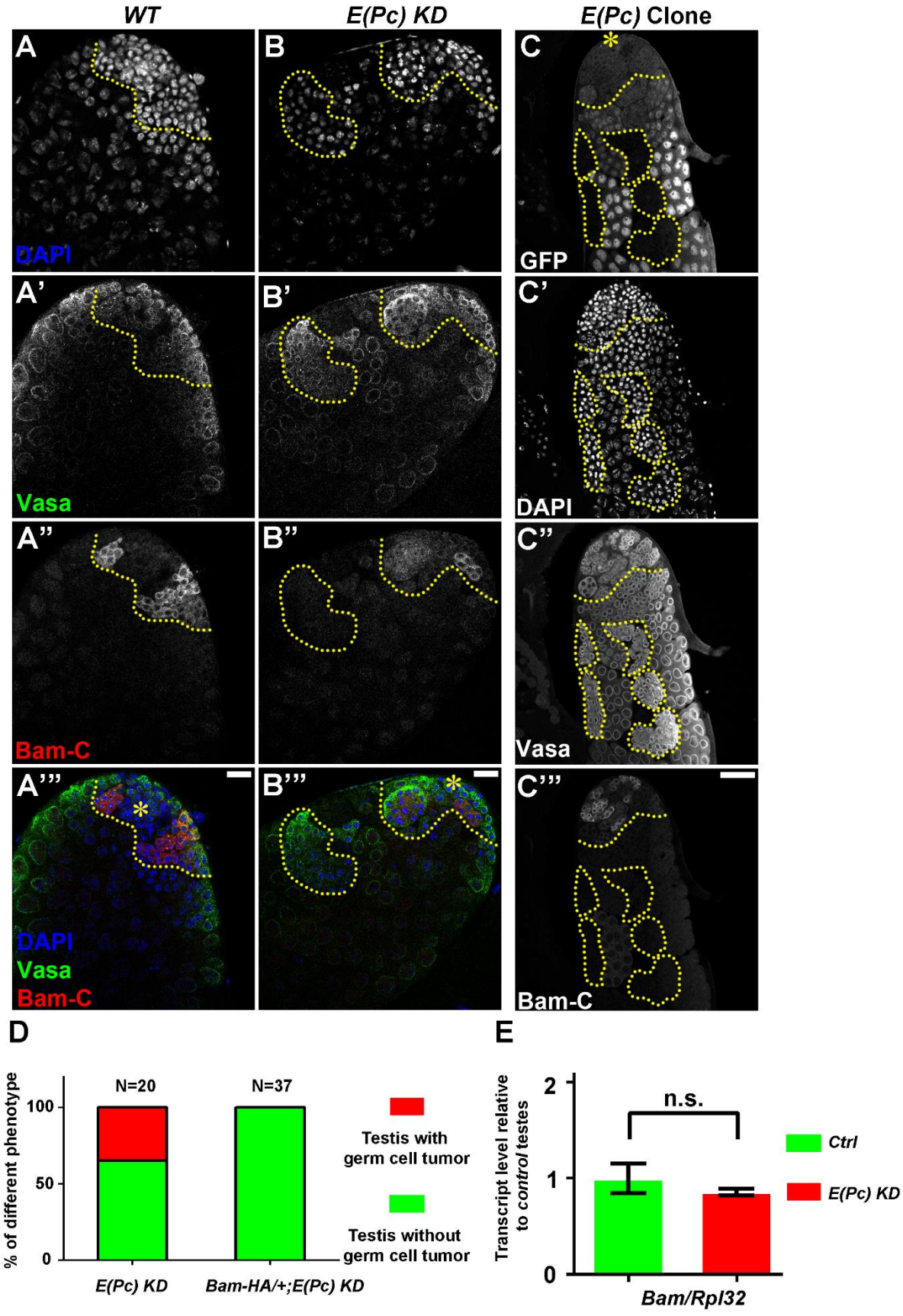

WT
E(Pc) KD
E(Pc) Clone
A
B
C
DAPI
GFP
A'
B'
C'
Vasa
DAPI
A''
B''
C''
Bam-C
Vasa
A'''
B'''
C'''
DAPI
Vasa
Bam-C
Bam-C
D
% of different phenotype
100
50
0
N=20
N=37
Testis with germ cell tumor
Testis without germ cell tumor
E(Pc) KD
Bam-HA/+;E(Pc) KD
E
Transcript level relative to control testes
2
1
0
n.s.
Ctrl
E(Pc) KD
Bam/Rpl32

Figure 2-6: E(Pc) in germ cells is required for expression of the differentiation factor Bam.

(**A-A"'**) In *WT* testes, DAPI bright (**A**) and Vasa-positive (**A'**) cells (yellow dashed line) represent early germ cells undergoing the transit amplifying stage. 4 to 16 stage SG cells, several cell diameters away from hub (asterisk), are Bam positive (**A",** red in **A'"**). (**B-B'"**) In *Tub-Gal80^{ts}, Nos>E(Pc) shRNA* (*E(Pc)* KD) testes, cyst with more than 16 germ cells (yellow outline) are found far away from the hub (asterisk). These small Vasa expressing germ cells (**B'**) have DAPI bright nuclei (**B**), characteristics of SG cells. However, no Bam signal (**B",** red in **B"'**) is detected for these overproliferating germ cells (yellow outline). Scale Bar: 20 μm. (**C-C'"**) GFP is used to identify *E(Pc)* null clones. Bam is expressed in 4-16 cell stage (yellow dashed line in C"'). SG clones (yellow outline) with more than 16 cells are far away from the hub (asterisk). They have DAPI bright nuclei (**C'**) and are relatively small based on Vasa staining (**C"**), hallmarks of SG cells. No Bam expression (**C"'**) is found in these *E(Pc)* SG clones (yellow outline). Scale Bar: 50 μm. (**D**) Percentage of testes with excess germ cells. (**E**) Quantitative RT-PCR of *bam* mRNA in *ctrl* (*Tub-Gal80^{ts}, Nos-Gal4*) and *E(Pc)* KD (*Tub-Gal80^{ts}, Nos>E(Pc) shRNA*) testes. Data shows mean ± SEM (standard error of the mean) based on three independent biological replicates. Transcript level in *ctrl* testes is set to 1.

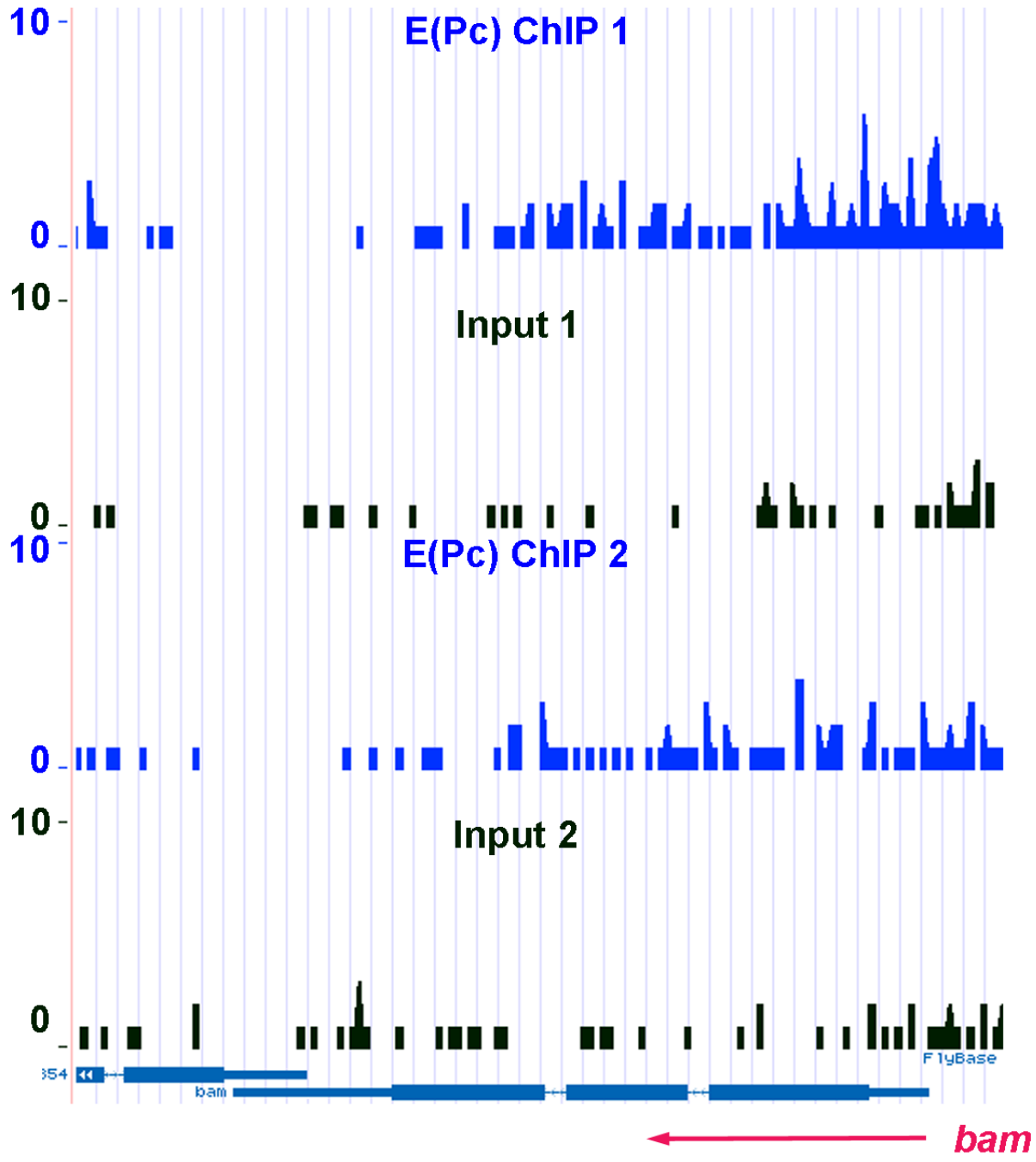


Figure 2-7: E(Pc) is not enriched at the genomic region of *bam*.

A genome browser snapshot of the *bam* gene region. Compared with the input control, E(Pc) GFP shows no enrichment at the genomic region of *CycB* in S2 cells. Two independent replicates are shown here.

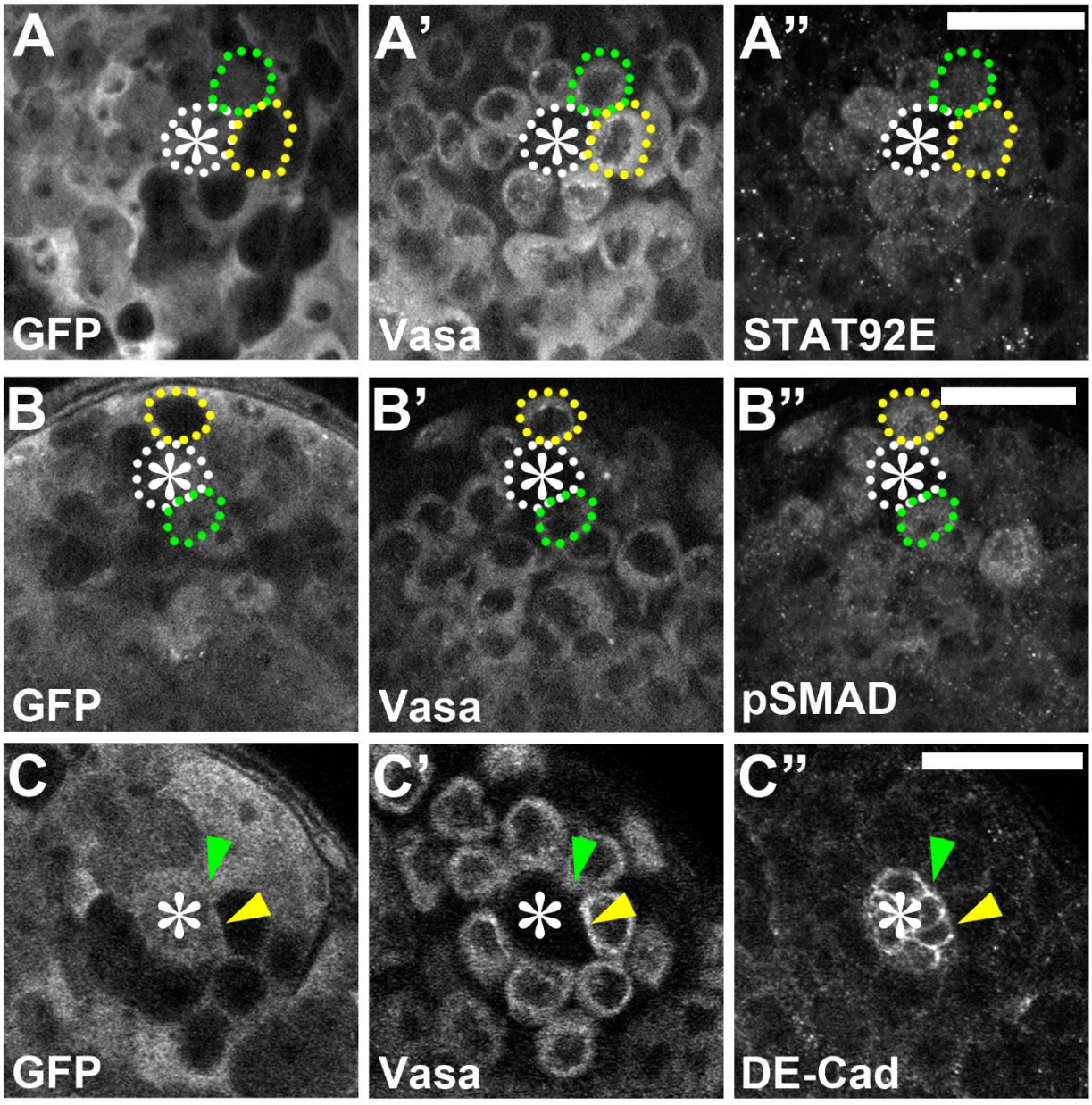
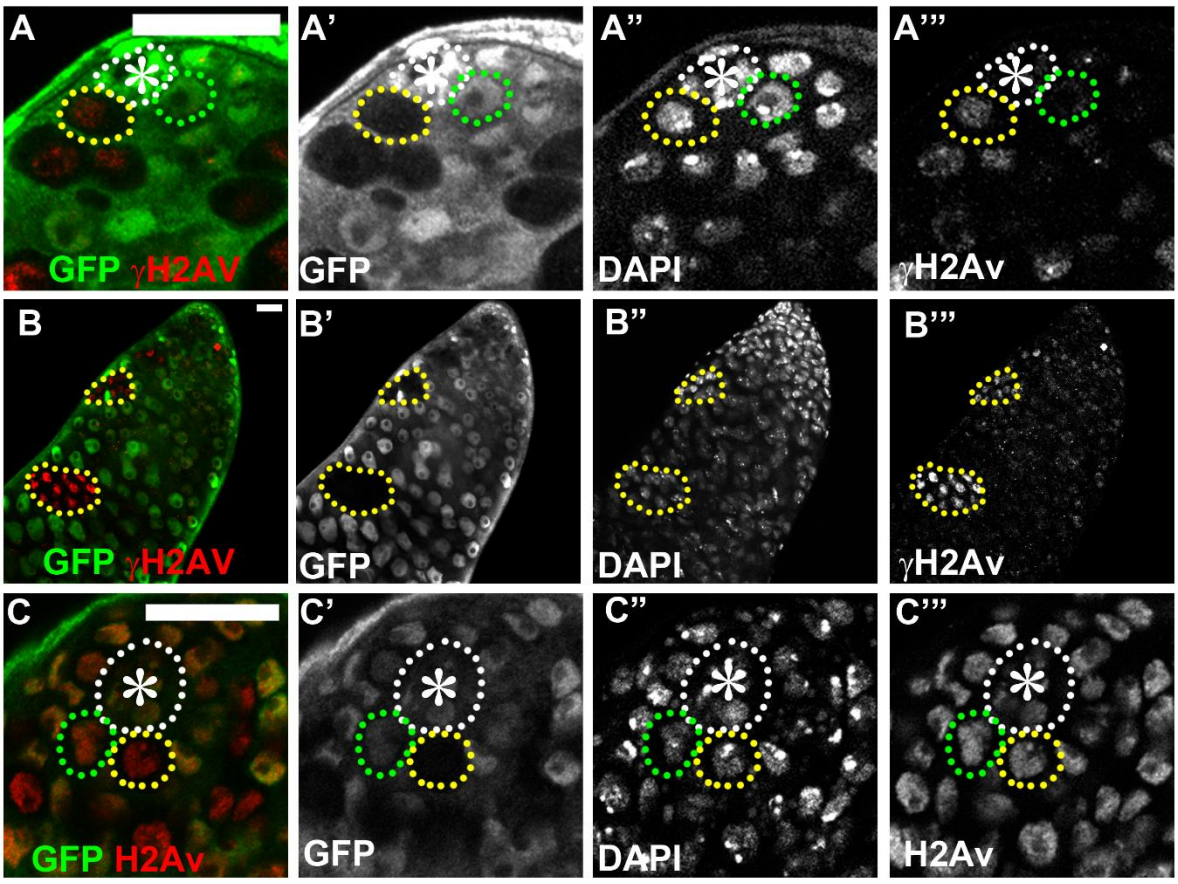
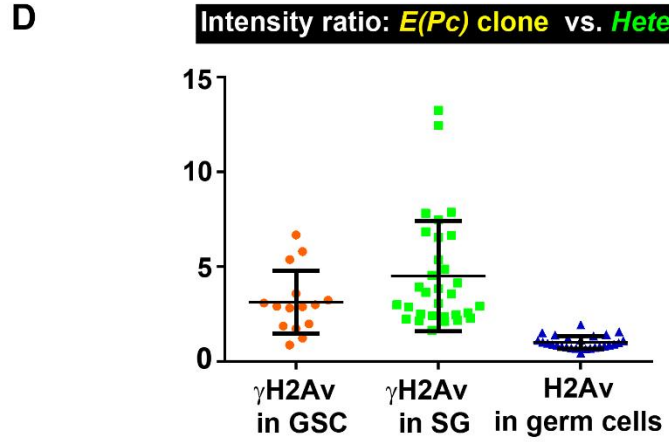


Figure 2-8: *E(Pc)* null GSC clones have unchanged JAK-STAT, BMP signaling activity, and DE-Cad level.

(**A-C"**) Apical tip of testes immunostained with GFP (**A**, **B**, and **C**) to identify *E(Pc)* null clones (yellow outline in **A**, **B**, yellow arrowhead in **C**) and *E(Pc)* heterozygous clones (green outline in **A**, **B**, green arrowhead in **C**). Vasa (**A'**, **B'** and **C'**) is used to identify germ cells. Immunostaining with STAT92E (**A"**), pSMAD (**B"**) and DE-Cad (**C"**) in *E(Pc)* null GSC clones and *E(Pc)* heterozygous clones show no difference. Hub: asterisk and white outline. Scale Bar: 20 μm.


A
A'
A''
A'''
GFP γH2AV
GFP
DAPI
γH2Av
B
B'
B''
B'''
GFP γH2AV
GFP
DAPI
γH2Av
C
C'
C''
C'''
GFP H2Av
GFP
DAPI
H2Av


D
Intensity ratio: E(Pc) clone vs. Heterozygous cells
15
10
5
0
γH2Av in GSC
γH2Av in SG
H2Av in germ cells

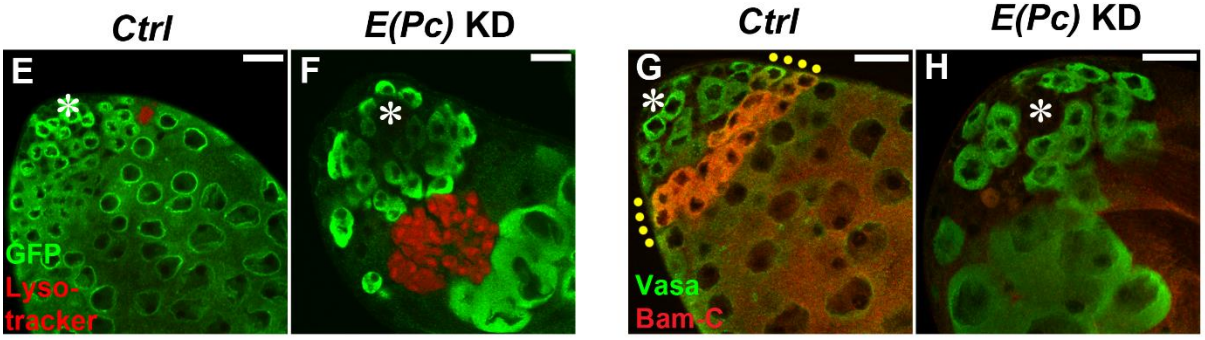

Ctrl
E(Pc) KD
Ctrl
E(Pc) KD
E
F
G
H
GFP
Lyso-tracker
Vasa
Bam-C

Figure 2-9: Germ cells without E(Pc) exhibit increased γH2Av and germ cell death.

(**A-C”’**) Apical tip of testes immunostained with GFP (**A’**, **B’** and **C’**) to identify GFP negative *E(Pc)* null clones and DAPI (**A”, B”** and **C”**) to show nuclei. Immunostaining with DNA DSB marker γH2Av (**A”’** and **B”’**), H2Av (**C”’**). Compared with *E(Pc)* heterozygous germ cells (green outline in **A”’**), *E(Pc)* null GSC clones (yellow outline in **A”’**) and SG clones (yellow outline in **B”’**) show increased γH2Av. In contrast, H2Av level is not changed between *E(Pc)* null GSC clone (yellow outline in **C”’**) and *E(Pc)* heterozygous GSC (green outline in **C”’**). Hub: asterisk and white outline. Scale Bar: 20 μm. (**D**) Quantification of the intensity of γH2Av and H2Av signal between *E(Pc)* germ cell clones and heterozygous germ cells. GSCs: $\gamma H2Av^{clone}/\gamma H2Av^{ctrl}=3.13 \pm 1.60$. SG: $\gamma H2Av^{clone}/\gamma H2Av^{ctrl}=4.49 \pm 2.86$. Germ cells: $H2Av^{clone}/H2Av^{ctrl}=1.00 \pm 0.33$. Data is shown as mean ± SD. Apical tip of *ctrl* (*Nos-Gal4*) (**E**) and *E(Pc)* KD (*Nos>E(Pc) shRNA*) testes (**F**) stained with the germ cell marker Vasa, cell death marker Lysotracker. (**G-H**) Apical tip of *ctrl* and *E(Pc)* KD testes stained with the differentiation factor Bam. In *ctrl* testes, Bam labels 4-to-16 spermatogonial cells (yellow dashed line). In *E(Pc)* KD testes, no Bam positive germ cells are found. Asterisk: Hub. Scale Bar: 20 μm.

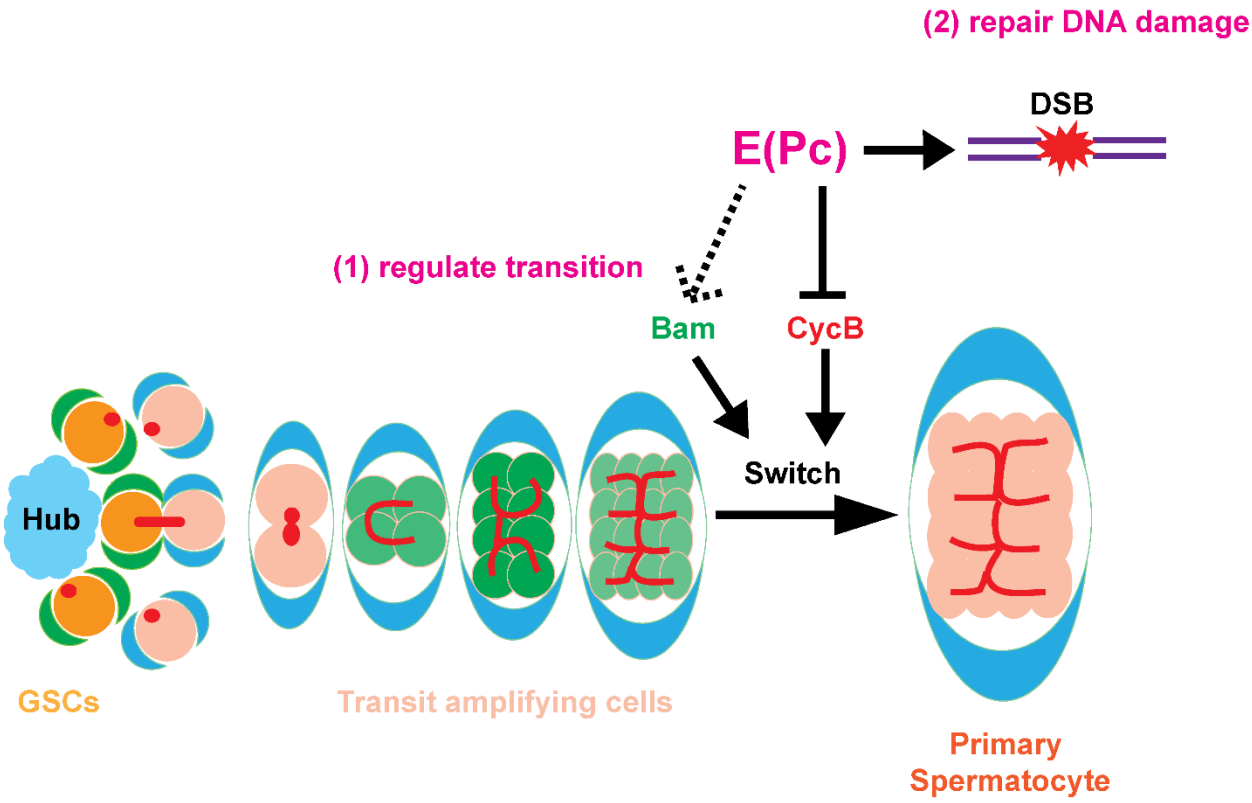


Figure 2-10: Model: E(Pc) is required for germ cell differentiation and maintenance.

During spermatogenesis, switch from the amplification stage to the spermatocyte stage requires reduced CycB and accumulation of Bam to a threshold level. E(Pc) can regulate the downregulation of CycB and activation of Bam to ensure the switch (**1**). In addition, efficient repair of DNA DSBs in the germ cells also requires E(Pc) function (**2**).

Chapter 3

Enhancer of Polycomb coordinates multiple signaling pathways to promote both cyst and germline stem cell differentiation in the *Drosophila* adult testis

INTRODUCTION

Adult stem cells under their physiological conditions are surrounded by other cell types and extracellular matrix. Recent studies have demonstrated a dynamic crosstalk between stem cells and their surrounding microenvironment termed as "niche" (Losick et al., 2011). Signaling molecules emanating from niche contribute to the balance between self-renewal and differentiation of adult stem cells, which are essential to maintaining tissue homeostasis and regeneration in response to injury. Therefore, a fundamental question in stem cell biology is how extrinsic cues and intrinsic factors cooperate to determine and maintain stem cell identity and activity.

Two adult stem cell lineages reside in the *Drosophila* adult testis: the germline stem cell (GSC) lineage and the cyst stem cell (CySC) lineage (**Fig.3-2A**). Both GSCs and CySCs attach to a group of post-mitotic somatic cells called hub cells and serve as a niche for each other. Both GSCs and CySCs undergo asymmetric cell divisions to produce one stem cell and one differentiated daughter cell in each lineage (Yamashita et al., 2003; Yamashita et al., 2010). The differentiation daughter cell in GSC lineage is called a gonialblast (GB), which subsequently undergoes a transit-amplifying stage with exactly four rounds of mitosis. Once the mitotic expansion is complete, germ cells enter the meiotic stage with an elongated G2 phase as spermatocytes, in which a robust gene expression program is initiated to prepare for meiotic divisions and spermatid differentiation. On the other hand, the differentiated daughter cell in CySC lineage becomes a cyst cell, which never divides again. Two cyst cells encapsulate synchronously dividing and differentiating germ cells and form a distinct germ cell cyst. Expansion of niche or niche-independent stem cells formation place stem cells at high risk of tumor formation (Clarke and Fuller, 2006). Conversely, dysfunction of stem cell due to impaired

niche is associated with compromised injury recovery, degenerative disease and aging (Rando, 2006). Studies using *Drosophila* gonads have unveiled conserved signaling and epigenetic regulation within the complex niche (Fuller and Spradling, 2007).

Drosophila testis has provided a paradigmatic system to study the communications between different stem cell lineages. For example, it has been shown that JAK-STAT and TGF-β signaling pathways are important for male GSC maintenance, through their intimate interactions with CySCs (Kiger et al., 2001; Leatherman and Dinardo, 2008, 2010; Tulina and Matunis, 2001). The ligand for the JAK-STAT signaling pathway Unpaired (Upd) is secreted by hub cells to activate the downstream transcription factor Stat92E in both CySCs and GSCs for their maintenance (Issigonis et al., 2009; Kiger et al., 2001; Leatherman and Dinardo, 2010; Singh et al., 2010; Tarayrah et al., 2015; Tulina and Matunis, 2001). In addition, the EGF signaling pathway has been shown to control cyst cells to encapsulate germ cells and allow their proper differentiation (Kiger et al., 2000; Sarkar et al., 2007; Schulz et al., 2004; Schulz et al., 2002; Tran et al., 2000). A protease called Stet acts in germ cells to cleave Spitz (Spi) ligand to stimulate EGF signaling in cyst cells (Schulz et al., 2002). Activation of the EGF signaling ensures encapsulation of germ cells by cyst cell and promotes germ cell differentiation (Hudson et al., 2013; Kiger et al., 2000; Matunis et al., 1997; Sarkar et al., 2007; Schulz et al., 2004; Schulz et al., 2002; Tran et al., 2000).

Even though most studies on germline and soma communication focused on signaling pathways while most work on chromatin regulators mainly addressed their cell-autonomous functions, recent studies demonstrate local signals cooperate with chromatin factors to regulate GSC maintenance and differentiation [reviewed by (Feng and Chen, 2015; Tarayrah and Chen, 2013)]. For example, JAK-STAT signaling in both GSCs and CySCs are positively regulated by

the nucleosome remodeling factor (NURF) for stem cell maintenance (Cherry and Matunis, 2010). The *Socs36E* gene encodes an inhibitor of the JAK-STAT signaling pathway, which is critical to maintaining the balance between GSCs and CySCs at the niche (Issigonis et al., 2009). Our previous studies show that a tri-methylation of histone H3 at Lys27 (H3K27me3)-specific histone demethylase encoded by *dUTX* gene acts upstream of and negatively regulates JAK-STAT signaling pathway through maintaining active transcription of *Socs36E* (Tarayrah et al., 2013). Moreover, EGF signaling pathway genes might be directly regulated by H3K27me3 methyltransferase Enhancer of Zeste [E(z)] in cyst cells to promote germ cell differentiation (Eun et al., 2014). However, identification of more crosstalk between signaling pathways and chromatin factors in CySC lineage is tempered by limited cyst cell number for any high-throughput experimental method such as Chromatin immunoprecipitation (ChIP) or protein co-immunoprecipitation. Thus, how cyst cell differentiation is regulated and how their differentiation is coordinated with neighboring germ cells remain unclear.

The *enhancer of Polycomb* [*E(Pc)*] gene is a putative Polycomb group (PcG) gene and is conserved from yeast to mammals, suggesting its crucial roles in regulating chromatin structure across species. The yeast homolog of *E(Pc)* was identified as a component of the NuA4 (nucleosome acetyltransferase of H4) histone acetyltransferase (HAT) complex (Boudreault et al., 2003; Chittuluru et al., 2011; Galarneau et al., 2000), which has been shown to contribute to the hyperacetylation state of H4 and H2A and stimulate transcription (Allard et al., 1999; Doyon and Cote, 2004; Nourani et al., 2001; Utley et al., 1998; Vignali et al., 2000). Abnormal activity of the human E(Pc) homolog called EPC1 has been shown to cause T-cell leukemia/lymphoma (Nakahata et al., 2009). However, the molecular and cellular mechanisms of the *in vivo* functions of E(Pc) have been elusive.

Here, we use the *Drosophila* adult testis as a model system to study functions of E(Pc) in endogenous adult stem cell lineages. We found that E(Pc) promotes cyst cell differentiation by downregulating CySC-enriched transcription factors Zfh-1 and Yan. Loss of such a repression by E(Pc) in CySC lineage blocked germ cell differentiation and caused early-stage germline tumors including both GSC-tumor and spermatogonial tumor. In addition, when *E(Pc)* is specifically knocked down in CySC lineage, overproliferating germ cells ectopically turn on expression of cyst cell markers such as Zfh-1 and Yan. When chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) was performed specifically in cyst cells, many components of key signaling pathways were identified as direct targets of E(Pc), suggesting its central role in coordinating crosstalk between CySC and GSC lineages. Furthermore, inactivation of the Tip60 histone acetyltransferase activity in CySC lineage led to defects resembling loss-of-function phenotypes of E(Pc), suggesting that they may act together *in vivo*. Thus, E(Pc) establishes the proper chromatin state in cyst cells to provide an instructive microenvironment to guide germ cell differentiation and protect germline identity.

RESULTS

E(Pc) downregulates CySC-enriched transcription factors to promote cyst cell differentiation

To understand *in vivo* functions of E(Pc) in the *Drosophila* adult testis, we first characterized E(Pc) expression pattern. Using a GFP-tagged genomic rescuing transgene (EXPERIMENTAL PROCEDURES), nuclear E(Pc)-GFP signal was detected in both germ cells and cyst cells throughout the adult testis (**Fig. 3-1A-A", B-B"**). The nuclear localization of E(Pc) is consistent with the prediction that E(Pc) is a chromatin regulator (Stankunas et al., 1998).

Because E(Pc) is required for early development and the null allele is lethal at embryonic or early larval stage (Sato et al., 1983), we studied functions of E(Pc) in adult testes using RNAi knockdown strategy (Mohr et al., 2014). When *E(Pc)* was knocked down in cyst cells using a cyst-cell-specific *Tj-Gal4* driver (Tanentzapf et al., 2007) paired with two different RNAi lines [*E(Pc) dsRNA* or *E(Pc) shRNA*, when phenotypes between these two lines are indistinguishable we call them in general as *E(Pc) RNAi*, see EXPERIMENTAL PROCEDURES], E(Pc)-GFP fusion protein level indicated by the GFP signal was greatly reduced compared with neighboring germ cells (**Fig. 3-1C-C"**). In CySC lineage of adult testis, two transcription factors are expressed in a spatiotemporally controlled manner: Zinc finger homeodomain protein 1 (Zfh-1), a transcription repressor with multiple zinc finger domains and a homeodomain, is highly expressed in CySCs and early cyst cells a few-cell-diameter away from the niche. It has been shown that Zfh-1 is required for CySC maintenance, which is also necessary for GSC maintenance (Leatherman and Dinardo, 2008). By contrast, another transcription factor Eye absent (Eya) is expressed in later stage cyst cells, which is required for cyst cell differentiation (Fabrizio et al., 2003). Immunostaining experiments showed a mutually exclusive pattern of Zfh-

1 and Eya with few cyst cells overlap (6.9±2.5) in control testis (N=44) (**Fig. 3-2B-B"**). By contrast, a significant number of cyst cells (more than 30) with both Zfh-1 and Eya immunostaining signals could be detected in 69% of *Tj>E(Pc) dsRNA* testes (N=32) and 63% of *Tj>E(Pc) shRNA* testes (N=35) , most likely due to ectopic Zfh-1 expression in later stage cyst cells (**Fig. 3-2C-C"**). Failure to downregulate Zfh-1 in later stage cyst cells suggests potential differentiation defects in *E(Pc)* knockdown cyst cells.

Based on microarray analysis (Terry et al., 2006) and RNA-seq data (Gan et al., 2010), a transcription repressor, *anterior open*, often referred as Yan is highly expressed in stem cell-enriched samples. Immunostaining experiments showed enriched Yan protein in CySCs and possibly the immediate daughter cells of CySCs in control testes (N=22) (**Fig. 3-2D**). By contrast, in 72% of *Tj>E(Pc) dsRNA* (N=18) and 74% of *Tj>E(Pc) shRNA* (N=35) testes, Yan-expressing cells expanded (**Fig. 3-2E**). Next, we used *hs-FLP; Actin-FRT-stop-FRT-Gal4, UAS-GFP; UAS- E(Pc) shRNA* fly strain to induce *E(Pc)* knockdown and GFP expression in the same cells *(hs*: *heat shock* promoter*, Actin: actin* promoter*).* We compared *E(Pc)*-knockdown and GFP-positive cells (arrows in **Fig. 3-2F-F", G-G"**) with the neighboring GFP-negative cells with normal E(Pc) (arrowheads in **Fig. 3-2F-F", G-G"**), ectopic expression of Zfh-1 (arrow in **Fig. 3-2F"**, N=13 cyst cells) or Yan (arrow in **Fig. 3-2G"**, N=11 cyst cells) was detected in GFP-positive cells with compromised E(Pc) activity.

In summary, we found that a chromatin factor E(Pc) acts in cyst cells to downregulate CySC-enriched transcription factors Zfh-1 and Yan, in order to promote cyst cell differentiation.

E(Pc) acts in cyst cells to promote germ cell differentiation and maintain germline identity

The CySC lineage has been thought to play a supportive role in germ cell differentiation. Cyst cells enclose germ cells and provide instructive signals for germline differentiation and survival (Zoller and Schulz, 2012). We found that knockdown of *E(Pc)* in cyst cells using *Tj-Gal4* led to an overpopulation of early-stage germ cells in 43% of *Tj>E(Pc) dsRNA* testes (N=40). Further reducing E(Pc) level using an EMS mutant $E(Pc)^{1}$ (Sinclair et al., 1998) as heterozygotes significantly enhanced the early germ cells overpopulation phenotype in *Tj>E(Pc) dsRNA* testes to 70% (N=20). Compared to the control testes (**Fig. 3-3A, A"**), knockdown of *E(Pc)* in cyst cells led to an overpopulation of early-stage germ cells, visualized by the expansion of cells with DAPI bright nuclei (**Fig. 3-3B, B"**) (Leatherman and Dinardo, 2008). Another marker for early-stage germ cells is the Notch receptor, which is present in early germ cells in control testes (Kiger et al., 2000) (**Fig. 3-3A', A"**). By contrast, Notch positive cells significantly increased in *E(Pc)* knockdown testis (**Fig. 3-3B', B"**). The expansion of Notch positive cells with DAPI bright nuclei (**Fig. 3-3B"**) often associate with proliferation-to-differentiation transition defects in the germline, as shown previously (Kiger et al., 2000; Leatherman and Dinardo, 2008; Tran et al., 2000).

We further analyzed the cellular properties of the overproliferative germ cells in either *Tj>E(Pc) dsRNA* (**Fig. 3-3**) or *Tj>E(Pc) shRNA* (**Fig. 3-4**) testes, representing two different RNAi lines for *E(Pc)* gene (EXPERIMENTAL PROCEDURES). In 12.5% of *Tj>E(Pc) dsRNA* testes (N=40, **Fig. 3-3C**) or 30% of *Tj>E(Pc) shRNA* testes (N=37, **Fig. 3-4A**), the overproliferative germ cells resembled GSC- or GB-tumor with round spectrosome structure intermingled with Zfh-1-positive CySC-like cells. In 30% of *Tj>E(Pc) dsRNA* testes (N=40, yellow dotted outline in **Fig. 3-3D-D'**) or 40% of *Tj>E(Pc) shRNA* testes (N=37, **Fig. 3-4B-B'**), the expanded germ cells were more like spermatogonial tumors with more than 16 Vasa-positive

cells within one cyst visualized by anti-Arm staining delineating the encapsulating cyst cells (**Fig. 3-3D, 3-4B**). However, different from the continuous fusome structure in spermatogonial cysts in wild-type or control testes (de Cuevas and Spradling, 1998; Hime et al., 1996; Lin et al., 1994), germ cells within one cyst had both dotted spectrosome (yellow arrowheads in **Fig. 3-3D, 3-4B-B'**) and branched fusome (yellow arrows in **Fig. 3-3D, 3-4B-B'**) structures, suggesting that these cells are not undergoing cell cycle in synchrony. This asynchrony was further confirmed when EdU incorporation assay was performed to label S-phase cells (Insco et al., 2009): for 54% single cysts (N=26), only a subset of overproliferative germ cells were labeled by EdU (arrow in **Fig. 3-3D'**). Bag-of-marbles (Bam) is an important differentiation factor expressed in 4- to 16-cell spermatogonia in wild-type (Gonczy et al., 1997; McKearin and Spradling, 1990) or control testes (**Fig. 3-5A-A'**). In all testes with spermatogonial tumor (45% of *Tj>E(Pc) dsRNA* testes, N=20; 57% of *Tj>E(Pc) shRNA* testes, N=21), Bam expression was detected in expanded germ cells (**Fig. 3-5B-B'**). In *bam* mutant testes, the transition from spermatogonia to spermatocyte is abolished and the testes are enriched with synchronously dividing spermatogonia (Gonczy et al., 1997; McKearin and Spradling, 1990). However, the presence of Bam (**Fig. 3-5B-B'**) and absence of continuous fusome structure (**Fig. 3-3D, 3-4B-B'**) suggest that these overproliferative germ cells in *E(Pc)* somatic knockdown testes have different cellular properties.

Even though these overproliferative cells were all positively stained with the germ cell marker Vasa in either *Tj>E(Pc) dsRNA* testes (**Fig. 3-3C, 3-3E, E"**) or *Tj>E(Pc) shRNA* testes (**Fig. 3-4A, 3-4B',3-4C, 3-4C"**), early stage cyst marker Zfh-1 was found to be ectopically expressed in 8% of *Tj>E(Pc) dsRNA* testes (N=49, **Fig. 3-3E'-E"**) or 11% of *Tj>E(Pc) shRNA* testes (N=37, **Fig. 3-4C'-C"**). Cell co-expressing Vasa and another early stage cyst marker Yan were also found in 13% of *Tj>E(Pc) dsRNA* testes (N=45, **Fig. 3-3F-F"**) or 12% of *Tj>E(Pc)*

shRNA testes (N=43, **Fig. 3-4D-D"**). Our previous studies demonstrate that E(z), a key PcG protein, is required in cyst cells to prevent germ cell from expressing Zfh-1, suggesting a non-cell-autonomous role of E(z) in antagonizing somatic cell fate in the germline (Eun et al., 2014). Interestingly, here compromising E(Pc) function showed similar germline phenotypes just like knocking down *E(z)* in the somatic gonadal cells. Furthermore, reducing *E(z)* activity either by an $E(z)^{731}$ null allele or a deficiency chromosome that uncovers *E(z)* region as heterozygotes, we observed higher penetrance of overproliferative early-stage germ cells shown as expanded DAPI bright region in *Tj>E(Pc) shRNA* testes (**Fig. 3-6A-D**). Similar loss-of-function phenotypes and genetic interactions between *E(Pc)* and *E(z)* are in accordance with the previous report that *E(Pc)* acts as an enhancer of PcG mutation phenotype (Sato et al., 1983).

Even though knocking down *E(Pc)* using the *Tj-Gal4* driver specific for all somatic gonadal cells including hub cells (**Fig. 3-2A**), knocking down *E(Pc)* using a hub cell-specific *upd-Gal4* driver (Boyle et al., 2007) did not lead to any detectable cellular defects (N=28, **Fig. 3-7**). These data demonstrate that E(Pc) acts in CySC lineage but not in hub cells to promote germ cell differentiation and antagonize somatic identity in the germline.

E(Pc) directly regulates multiple signaling pathway components and the CySC self-renewal factor Zfh-1

In order to fully understand the molecular mechanisms how E(Pc) functions in cyst cells to promote cellular differentiation, a chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) strategy was developed to profile the direct targets of E(Pc) specifically in CySC lineage: in *Tj>E(Pc) shRNA* testes, a *GFP*-tagged *E(Pc) cDNA* transgene was expressed using the same *Tj-Gal4* driver. We found not only the *E(Pc)* knockdown

phenotypes (**Fig. 3-2, Fig. 3-3, Fig. 3-4**) were fully rescued in *Tj>E(Pc) GFP, E(Pc) shRNA* testes (N=137), but also the GFP-tagged E(Pc) was only detectable in CySC lineage (**Fig. 3-8A**). Therefore this genetic background provides a unique opportunity to immunoprecipitate E(Pc)-bound chromatin in CySC lineage using the ChIP-grade GFP antibody (Kumar and Wigge, 2010).

We next analyzed the ChIP-seq data to identify direct target genes of E(Pc) in CySC lineage. When all target genes were plotted over a -1-kb to +4-kb region with respect to the transcription start sites (TSSs), enrichment of E(Pc) could be detected within a 600-bp region upstream of TSSs (**Fig. 3-8B**), consistent with the prediction that E(Pc) is a chromatin factor regulating transcription of target genes. Using MACS2 with default setting and P-value cutoff of 1e-5, we identified 4,698 E(Pc)-bound genes in cyst cells in adult testes. Using GO term enrichment test (Eden et al., 2007; Eden et al., 2009) to further analyze the direct target genes of E(Pc), we found that signaling pathway components, genes responsible for DNA damage checkpoint, and genes encoding histone modifying enzymes represent the top three categories (**Fig. 3-8C**). In signaling pathway category, genes associated with epidermal growth factor receptor (Egfr) signaling pathway, JAK-STAT cascade, Wnt and Notch signaling pathways are significantly enriched ($P<0.001$) with E(Pc) targets.

In addition, consistent with overpopulated Zfh-1-positive cells in *Tj>E(Pc) RNAi* testes (**Fig. 3-2C, 3-2F"**), enrichment of E(Pc) was found at the endogenous *zfh-1* gene locus (**Fig. 3-8D**). This result suggests that E(Pc) directly binds to and downregulates *zfh-1* expression in cyst cells to promote their differentiation. Consistently, when RNA-seq was performed to compare transcriptomes between *Tj>E(Pc) shRNA* testes and *Tj-Gal4* control testes, a significant upregulation of *zfh-1* was detected in *Tj>E(Pc) shRNA* testes (1.64-fold upregulation, $P< 0.01$).

By contrast, no E(Pc) enrichment was detected at the endogenous *eya* gene locus (**Fig. 3-8E**), consistent with the data that no change in *eya* mRNA level was identified based on RNA-seq and no change in Eya expression could be detected in *Tj>E(Pc) RNAi* testes (**Fig. 3-2C', 3-2F'**). For another early stage cyst cell marker Yan, although E(Pc) enrichment at *yan* locus did not pass the cutoff using peak calling algorithm (Zhang et al., 2008), local enrichment could be detected (**Fig. 3-8F**), indicating *yan* is a potential direct target of E(Pc) in CySC lineage.

If E(Pc) acts as a transcriptional repressor to downregulate *zfh-1* expression, overexpression of E(Pc) may lead to decreased Zfh-1. Because Zfh-1 is required for CySC self-renewal and GSC maintenance (Leatherman and Dinardo, 2010), reduction of *zfh-1* might result in loss of both CySCs and GSCs. Indeed, when E(Pc) was overexpressed in CySC lineage using *Tj>E(Pc) GFP*, a significant reduction of Zfh-1 positive cells was observed (**Fig. 3-9A-B**). Moreover, both CySCs and GSCs showed significant decreases in *Tj>E(Pc) GFP* testes (**Fig. 3-9C-D**). In summary, E(Pc) is both necessary and sufficient to repress *zfh-1* expression in CySC lineage.

E(Pc) acts in synergy with EGF signaling pathway to promote cellular differentiation in both CySC and GSC lineages

The top category of E(Pc)-controlled target genes are signaling pathway components (**Fig. 3-8C**), suggesting its important roles in coordinating somatic and germline cellular differentiation pathways. A previous RNAi screen using *Drosophila* S2R+ cells has identified E(Pc) as a positive regulator of receptor tyrosine kinase and ERK signaling pathway (Friedman and Perrimon, 2006). Indeed, in our ChIP-seq data, local enrichment of E(Pc) could be detected at endogenous *yan* locus (**Fig. 3-8F**). Consistent with the transcriptional repressor function of

E(Pc), Yan was upregulated in *E(Pc)* knockdown cyst cells (**Fig. 3-2E, 3-2G"**). Because Yan functions as an antagonist of the EGF signaling (Lai and Rubin, 1992; Rebay and Rubin, 1995), E(Pc) could be a positive regulator of EGF signaling in CySC lineage. We next studied potential synergistic interactions between E(Pc) and EGF signaling pathway.

The EGFR signaling pathway has been shown to control cyst cells to encapsulate germ cells and allow their proper differentiation (Chen et al., 2013a; Hudson et al., 2013; Kiger et al., 2000; Parrott et al., 2012; Sarkar et al., 2007; Schulz et al., 2004; Schulz et al., 2002; Tran et al., 2000). Indeed, knocking down of *E(Pc)* in cyst cells resulted in phenotypes resembling those caused by loss-of-function of the EGF signaling pathway components. For example, it has been reported when EGF signaling is compromised, germ cells have differentiation defects (Kiger et al., 2001; Schulz et al., 2002; Tran et al., 2000) and divide asynchronously (Hudson et al., 2013), similar to those germline phenotypes in *Tj> E(Pc) RNAi* testes (**Fig. 3-3D-D', 3-4B-B'**). Using the Vein-LacZ reporter as a read-out of EGF signaling activity (Golembo et al., 1999; Kiger et al., 2001; Wasserman and Freeman, 1998; Wessells et al., 1999), we found absence of this reporter expression in early stage Zfh-1-expressing cyst cells (yellow arrowhead in **Fig. 3-10A-A"**) but robust expression in differentiated cyst cells (yellow arrows in **Fig. 3-10A-A"**), suggesting increased EGF signaling activity during normal cyst cell differentiation, as reported previously (Hudson et al., 2013). However, in *Tj>E(Pc) shRNA* testes, Vein-LacZ expression was almost undetectable in later stage cyst cells (yellow arrows in **Fig. 3-10B-B"**), indicating compromised EGF signaling activity due to *E(Pc)* knockdown. When the intensity of Vein-LacZ signal in later stage cyst cells (yellow arrows in **Fig. 3-10A-A", 3-10B-B"**) was quantified in *Tj-Gal4* and *Tj>E(Pc) shRNA* testes (EXPERIMENTAL PROCEDURES), a significant difference was identified (**Fig. 3-10C**). Moreover, consistent with the potential synergistic interactions

between *E(Pc)* and EGF signaling, an $Egfr^{f2}$ null allele enhanced the germline differentiation defects in *Tj>E(Pc) dsRNA* testes (**Fig. 3-10D**).

Yan is an ETS domain transcriptional repressor which antagonizes EGF signaling pathway (Lai and Rubin, 1992) and inhibits cellular differentiation (Rebay and Rubin, 1995). Yan is normally detectable restrictively in CySCs and possibly the immediate daughter cells of CySCs (**Fig. 3-2D**), but becomes ectopically upregulated in *E(Pc)*-knockdown cells (**Fig. 3-2E, 3-2G"**), suggesting that the normal function of E(Pc) is to repress Yan expression to promote cyst cell differentiation. In addition, a constitutively active form of Yan (Yan^{CA}), when expressed in cyst cells using the *Tj-Gal4* driver, resulted in similar phenotypes as observed in *Tj>E(Pc) RNAi* testes (**Fig. 3-2C, 3-3C, 3-3D, 3-4A, 3-4B-B'**): (1) Zfh-1-positive cells were over-populated (**Fig. 3-10E'**) in all *Tj>* Yan^{CA} testes (N=21); (2) over-proliferative germ cells were detected as GSC- or GB-like tumors (yellow outline, **Fig. 3-10E-E"**) in all *Tj>* Yan^{CA} testes (N=21) and spermatogonial tumors (white outline, **Fig. 3-10E-E"**) in 90% *Tj>* Yan^{CA} testes (N=21). Finally, a null allele yan^{IP} (Rogge et al., 1995) acted as a strong suppressor of the germline differentiation defects in *Tj>E(Pc) shRNA* testes (**Fig. 3-10F**), consistent with the hypothesis that part of the *E(Pc)* knockdown phenotype is due to upregulated expression of Yan.

Activated EGF signaling has been shown to induce phosphorylated active MAP kinase (dpERK) to enter the nucleus in order to regulate transcription of target genes (Chen et al., 2013a; Gabay et al., 1997; Kiger et al., 2000). To further understand how E(Pc) regulates EGF signaling, we characterized expression level and localization of dpERK in *E(Pc)* knockdown cyst cells. We induced *E(Pc)* knockdown and GFP expression in the same cells using the strategy discussed above (**Fig. 3-2F-G"**). When *E(Pc)*-knockdown and GFP-positive cells (arrow in **Fig. 3-10G**) were compared with the neighboring GFP-negative cells with normal E(Pc) (arrowhead

in **Fig. 3-10G**), no difference could be identified at either the expression level or the subcellular localization of dpERK (**Fig. 3-10G'**). These data indicate that E(Pc) may act in parallel with or downstream of dpERK to regulate the chromatin state of target genes responsive to EGF signaling.

E(Pc) represses JAK-STAT signaling to promote cyst cell differentiation

The JAK-STAT signaling pathway has been shown to play prominent roles in regulating self-renewal of both CySCs and GSCs (Hasan et al., 2015; Issigonis et al., 2009; Kiger et al., 2001; Leatherman and Dinardo, 2008, 2010; Stine et al., 2014; Tulina and Matunis, 2001). The Upd ligand is secreted locally from hub cells and acts through the Domeless receptor to activate the Janus kinase Hopscotch and phosphorylate the STAT92E transcription factor, which is subsequently translocated to the nucleus in order to activate target genes (Brown et al., 2001; Zeidler et al., 2000). Our ChIP-seq data identified significant enrichment of E(Pc) at the genomic loci of multiple JAK-STAT pathway genes, including *domeless*, *hopscotch* and *stat92E* (**Fig. 3-11A**), suggesting that E(Pc) might directly regulate JAK-STAT signaling pathway activity by targeting several key components.

To assess JAK-STAT signaling activity in cyst cells, we used a 2X STAT-GFP reporter (Bach et al., 2007; Hombria and Sotillos, 2013; Sinden et al., 2012; Tarayrah et al., 2013). This reporter has two copies of STAT92E DNA binding sites from *Socs36E* genomic locus which have been inserted upstream of the GFP reporter. In control testes (N=27), the GFP signal is only detectable in CySCs localized one cell diameter away from the hub region (arrowheads in **Fig. 3-11B-B"**), but not in differentiated cyst cells labeled with Eya immunostaining (arrows in **Fig. 3-11B-B"**). By contrast, in 77% of *Tj>E(Pc) shRNA* testes (N=52), Eya-positive cells showed

robust GFP signal (arrows in **Fig. 3-11C-C"**), indicating active JAK-STAT signaling in these later stage cyst cells. Ectopic JAK-STAT activity in cyst cells with compromised E(Pc) supports the hypothesis that E(Pc) represses JAK-STAT signaling to promote cyst cell differentiation. Consistent with this hypothesis, another JAK-STAT signaling target gene Zfh-1 (Leatherman and Dinardo, 2008) also showed ectopic expression in later stage cyst cells in *Tj>E(Pc) RNAi* testes (**Fig. 3-2C, F"**). In summary, these data support that E(Pc) directly represses JAK-STAT signaling activity in CySC lineage in order to promote cyst cell differentiation.

E(Pc) acts in synergy with Tip60 whose functions in cyst cells depend on its histone acetyltransferase activity

The *Drosophila* E(Pc) was identified as a component of the Tip60 histone acetyltransferase complex in S2 cells (Kusch et al., 2004). Biochemical experiments demonstrate that Tip60 acetylates H4 and H2A; and such an activity is conserved from yeast (Boudreault et al., 2003) to human (Ikura et al., 2000).

In order to examine how Tip60 and E(Pc) cooperate in CySC lineage, we first examined loss-of-function phenotypes of Tip60 using two strategies: a *Tip60 RNAi* transgene (Zeng et al., 2015) (*Tj>Tip60 RNAi*) and a *Tip60* dominant negative form (*Tj>Tip60*^{E431Q}) (Lorbeck et al., 2011), both driven by the same *Tj-Gal4* in CySC lineage as used in *E(Pc)* knockdown experiments (**Fig.3-2,3-3**). We found both strategies led to defects similar to phenotypes characterized in *Tj>E(Pc) RNAi* testes: (1) ectopic Zfh-1 expression could be detected in 74% of *Tj>Tip60 RNAi* (N=70, **Fig. 3-12A'**) and 77% of *Tj>Tip60*^{E431Q} (N=52, **Fig. 3-12B'**) testes, suggesting failure in prompt downregulation of Zfh-1 in later stage cyst cells. The ectopic expression of Zfh-1 led to cyst cells co-expressing early-stage marker Zfh-1 and later stage

marker Eya in both *Tj>Tip60 RNAi* (yellow arrows, **Fig. 3-12A'-A"'**) and *Tj>Tip60^{E431Q}* (yellow arrows, **Fig. 3-12B'-B"'**) testes. (2) In 46% of *Tj>Tip60 RNAi* (N=70, **Fig. 3-12A**) and 50% of *Tj>Tip60^{E431Q}* (N=52, **Fig. 3-12B**) testes, expansion of germ cells with DAPI bright nuclei was detected. Further characterization of the over-proliferative germ cells showed early stage germline tumor (**Fig. 3-12C**) in 8% and spermatogonial tumor (white dotted outline, **Fig. 3-12D**) in 28% of *Tj>Tip60 RNAi* testes (N=60). Similar early stage germline tumor (**Fig. 3-12E**) and spermatogonial tumor (white dotted outline, **Fig. 3-12F**) were found in 8% and 47% *Tj>Tip60^{E431Q}* testes (N=38), respectively. (3) Overproliferative cells with both germline marker Vasa and early cyst cell marker Zfh-1 could be detected in 18% of *Tj>Tip60 RNAi* testes (N=60, **Fig. 3-12G-G"**) and 19% of *Tj>Tip60^{E431Q}* testes (N=21, **Fig. 3-12I-I'**). Cells co-expressing Vasa and another early stage cyst cell marker Yan were also observed in 10% of *Tj>Tip60 RNAi* testes (N=60, **Fig. 3-12H-H"**) and 29% of *Tj>Tip60^{E431Q}* testes (N=38, **Fig. 3-12J-J"**). Because the E431Q mutation in the *Tip60^{E431Q}* transgene abolishes the HAT activity (Halkidou et al., 2004; Patel et al., 2004) of Tip60, similar phenotypes between *Tj>Tip60 RNAi* and *Tj>Tip60^{E431Q}* testes demonstrate that the function of Tip60 in CySC lineage relies on its histone acetyltransferase activity. In summary, both cyst cell and germline defects in either *Tj>Tip60 RNAi* or *Tj>Tip60^{E431Q}* testes are similar to those in *Tj>E(Pc) RNAi testes* (**Fig. 3-2B-E**, **Fig. 3-3** and **Fig. 3-4**), suggesting that E(Pc) and Tip60 may act together in cyst cells to regulate cyst cell differentiation cell-autonomously, as well as coordinate germ cell differentiation and maintain germline fate non-cell-autonomously.

To further explore the potential synergistic functions between E(Pc) and Tip60, we tested genetic interactions between them: using a permissive condition for RNAi knockdown experiments (Duffy, 2002; Staehling-Hampton et al., 1994; Wilder and Perrimon, 1995), no

germline tumor was detected in *Tj>Tip60 RNAi* testes (N=36) and such a phenotype was only detected in 13% of *Tj>E(Pc) shRNA* testes (N=30). By contrast, 31% of *Tj>Tip60 RNAi*; *E(Pc) shRNA* testes (N=39) showed germline tumor. The higher penetrance of germline tumor phenotype by knocking down both *Tip60* and *E(Pc)* suggest that these two genes may act in synergy in CySC lineage in regulating germ cell differentiation. Noticeably, overexpression of Tip60 also led to significant reduction of Zfh-1-positive cells (**Fig. 3-13A**), similar to E(Pc) overexpression phenotype (**Fig. 3-9A**). Because Zfh-1 is required for CySC self-renewal and GSC maintenance, there was a significant decrease of GSCs in *Tj>Tip60* testes (**Fig. 3-13B**), similar to *Tj>E(Pc)* testes (**Fig. 3-9C-D**). In summary, Tip60 resembles E(Pc) in its necessary and sufficient roles to repress *zfh-1* expression in CySC lineage.

DISCUSSIONS

In the testicular niche of *Drosophila*, the CySC lineage has been thought to play a supportive role in germ cell proliferation and differentiation. But how the maintenance and differentiation of CySC lineage are regulated as well as how this regulation is coordinated with the germline lineage remains poorly understood. Here we show that a PcG component E(Pc) is required for CySC differentiation, and in turn promotes germ cell differentiation. E(Pc) is also required to maintain germ cell identity (**Fig. 3-14A**). Loss of this critical chromatin regulator in CySC lineage leads to germ cell overproliferation and expression of several somatic cell markers tested (**Fig. 3-14B**). To understand the molecular mechanism of E(Pc), we performed ChIP-seq to specifically identify E(Pc)-bound targets in somatic gonadal cells of *Drosophila* testis. E(Pc) is found to enrich at many important genes known to be functional in somatic gonadal cells. In particular, E(Pc)-binding genes are enriched with signaling pathway components. Analyses of

E(Pc) targets not only confirm some known regulatory mechanisms coordinating CySC and GSC lineages, such as EGF signaling; but also shed light on some new mechanisms. For example, we identified that the *zfh-1* gene required for CySC maintenance and function is a direct target of E(Pc). Consistent with published biochemical results (Kusch et al., 2004), our studies also revealed that in cyst cells E(Pc) works with Tip60 in a HAT-dependent manner to promote cyst cell differentiation. Our finding of the instructive roles of cyst cells in guiding germ cell differentiation is consistent with the previous finding that ablation of cyst cells leads to germ cell differentiation defects (Lim and Fuller, 2012).

E(Pc) is a unique Polycomb group gene

The PcG complex plays critical roles in the cell decision between maintaining proliferating precursor state and initiating the terminal differentiation program. The PcG complexes may use multiple mechanisms to silence transcription, for example, by making the chromatin more compact (Francis et al., 2004), or by interfering with transcription initiation (Dellino et al., 2004; Wang et al., 2004) and/or elongation (Stock et al., 2007). It is generally agreed that PcG complexes employ epigenetic mechanisms that alter chromatin state to repress gene expression (Surface et al., 2010). For example, the PcG component E(z) enzyme generates the H3K27me3 mark (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002).

In *Drosophila*, *E(Pc)* gene was proposed to be a PcG component because *E(Pc)* mutants act as strong enhancers of *Pc* homeotic phenotype (Sato et al., 1983; Stankunas et al., 1998). In addition, many E(Pc) binding sites at polytene chromosomes overlap with other PcG components (Stankunas et al., 1998). However, *E(Pc)* is an unusual member of *PcG* family genes because it

has dual roles as an enhancer of *Pc* homeotic phenotype and a suppressor of position effect variegation (Sato et al., 1983; Sinclair et al., 1998; Stankunas et al., 1998). Interesting, among all *PcG* genes, only *E(Pc)* and *E(z)* have these dual roles (Laible et al., 1997; Stankunas et al., 1998), suggesting that they may have similar functions. Consistently, our previous studies have revealed that knocking down *E(z)* in somatic gonadal cells leads to phenotypes (Eun et al., 2014) similar to those induced by loss-of-function of *E(Pc)* shown in this study. Moreover, analyses of E(Pc) targets in somatic gonadal cells suggest that E(Pc) might directly regulate E(z) expression (**Fig. 3-15A, 3-15B, 3-15C**). Up to date most studies of PcG have focused on the mechanisms by which PcG proteins repress target gene expression, but not on the regulation of PcG genes themselves. Therefore, these results showing that E(Pc) may act as an upstream chromatin factor to regulate *E(z)* and other PcG genes may shed light on how these important developmental regulators are themselves regulated.

In addition to the genetic data showing association with PcG complex, E(Pc) has also been shown by biochemical data to be a component of the Tip60 histone acetyltransferase complex (Kusch et al., 2004). We found that knockdown of *Tip60* resulted in similar phenotype compared to E(Pc) phenotype, in line with a previous study showing that Tip60 represses differentiation gene expression to maintain pluripotency of mouse embryonic stem cells (Fazzio et al., 2008). Interestingly, our ChIP-seq assay indicated that Tip60 is also a potential direct target of E(Pc) (**Fig. 3-15D**). In this scenario, E(Pc) likely acts as an activator to promote *Tip60* expression based on the similar phenotypes between *Tip60* and *E(Pc)* knockdown and synergistic genetic interaction between E(Pc) and Tip60.

Our genomic and genetic data show that E(Pc) can act as both a transcriptional repressor and activator, and both functions are important to regulate CySC differentiation to coordinate

with GSC differentiation. *Drosophila* E(Pc) has four conserved domains, namely E(Pc)A, E(Pc)B, E(Pc)C and glutamine enriched region (Stankunas et al., 1998). Structure and function studies of human EPC demonstrated that EPcA region activates transcription, while the rest regions are capable of repressing gene expression (Shimono et al., 2000; Tezel et al., 2002), in accordance with our findings of the dual-functional roles of E(Pc) in regulating target gene expression.

New germline defects due to loss-of-function of a chromatin regulator in the neighboring somatic gonadal cells

Even though compromising E(Pc) in CySC lineage leads to CySC differentiation defects, the most prominent phenotypes are detected in the germline. Germ cells in *Tj>E(Pc) RNAi* testes have interesting new phenotypes: first, overproliferative germ cells divide asynchronously, which is different from previous identified spermatogonial tumors in *bam* or *benign gonial cell neoplasm* (*bgcn*) mutant testes. In the absence of Bam or Bgcn, the transition from spermatogonia to spermatocyte is abolished and the testes are enriched with synchronously dividing spermatogonia (Gonczy et al., 1997; McKearin and Spradling, 1990). However, the presence of Bam (**Fig. 3-5B-B'**) and absence of continuous fusome structure (**Fig. 3-3D, 3-4B-B'**) suggest that these overproliferative germ cells in *Tj>E(Pc) RNAi* testes have different cellular properties. In addition, the overproliferative germ cells do not resemble expanded early-stage germ cells upon hyperactivation of the JAK-STAT signaling pathway in *Drosophila* testis (Kiger et al., 2001; Leatherman and Dinardo, 2008; Tulina and Matunis, 2001). In fact, the overproliferative germ cells in *Tj>E(Pc) RNAi* testes has features resembling both spermatogonial tumors and GSC- or GB-like tumors (**Fig.3-3** and **Fig. 3-4**).

Second, germ cells with compromised E(Pc) in the neighboring somatic gonadal cells ectopically turn on early-stage cyst cell markers such as Zfh-1 and Yan, indicating E(Pc) acts in cyst cells to prevent germ cells from taking somatic cell fate. The dichotomy between germline and soma represents the earliest lineage specification among many metazoan organisms. In multiple model organisms, including *C. elegans* and *Drosophila*, germ cell identity is determined by maternally loaded germ granules (Ephrussi et al., 1991; Hay et al., 1988; Raz, 2003; Strome and Wood, 1983; Wang and Lehmann, 1991; Wylie et al., 1985). After specification, protection of germline fate requires both chromatin state and cytoplasmic factors in germ cells (Asaoka-Taguchi et al., 1999; Deshpande et al., 2005; Deshpande et al., 1999; Hanyu-Nakamura et al., 2008; Martinho et al., 2004; Nakamura et al., 1996; Nakamura and Seydoux, 2008; Strome and Updike, 2015). The previous study identified that the PcG component E(z) has a non-cell autonomous role in repressing somatic fate of germ cells in adult *Drosophila* testis (Eun et al., 2014). Here we identified that both E(Pc) and Tip60 have similar roles in cyst cells to maintain germline identity in a non-cell autonomous manner, indicating that they might act with E(z) in regulating a critical signaling pathway to keep germline identity throughout adulthood. These results also emphasize the important roles of the somatic gonadal cells in the protection of germline from taking somatic cell fate to ensure proper differentiation into functional gametes.

E(Pc) is a potential master regulator in regulating multiple signaling pathways to coordinate differentiation pathways between CySC and GSC lineages

Even though previous studies suggest the somatic gonadal cells control germ cell differentiation and maintain germline identity through multiple signaling pathways (Eun et al., 2014; Kiger et al., 2001; Kiger et al., 2000; Leatherman and Dinardo, 2010; Li et al., 2014; Lim

and Fuller, 2012; Matunis et al., 2012; Qian et al., 2014; Stine et al., 2014; Tran et al., 2000; Tulina and Matunis, 2001; Zoller and Schulz, 2012), it is unclear how these signaling pathways are coordinated. Here our ChIP-seq results reveal that E(Pc) is enriched at key components of multiple signaling pathways that are known to be important in regulating germ cell function. For example, the EGF signaling pathway has been shown to regulate cyst cells in encapsulating germ cells and promoting their proper differentiation in *Drosophila* testis (Chen et al., 2013a; Hudson et al., 2013; Kiger et al., 2000; Parrott et al., 2012; Sarkar et al., 2007; Schulz et al., 2004; Schulz et al., 2002; Tran et al., 2000). Here we found EGF antagonist Yan is highly enriched in CySCs but decreased dramatically in later stage cyst cells, which is repressed by E(Pc) during CySC differentiation. Consistently, the expression of Vein which is downstream of EGF signaling is compromised in *Tj>E(Pc) RNAi* testes (**Fig. 3-10B-B", 3-10C**), consistent with ectopic Yan and suppressed EGF signaling in the absence of E(Pc). Recently, decreased EGF signaling has been shown to induce extra germ cell division out of synchrony (Hudson et al., 2013). Similar asynchronous overproliferation of germ cell upon compromising either E(Pc) function or EGF signaling activity support the hypothesis that E(Pc) acts in synergy with EGF signaling pathway, probably through direct regulation of the chromatin state at the endogenous *yan* genomic locus.

In addition, E(Pc) was found to be enriched at multiple JAK-STAT pathway components. Different from increased EGF signaling activity during CySC differentiation, high JAK-STAT signaling activity is detected in early stage cells of both CySC and GSC lineages (Issigonis et al., 2009; Sheng et al., 2009b; Sinden et al., 2012; Tarayrah et al., 2015; Wawersik et al., 2005). Hyperactivation of JAK-STAT signaling in either CySC or GSC lineage is sufficient to block cellular differentiation and results in tumors with CySC- and GSC-like features (Kiger et al., 2001; Leatherman and Dinardo, 2008; Tulina and Matunis, 2001). In this scenario, E(Pc) might

downregulate JAK-STAT signaling to promote CySC differentiation by directly repressing expression of key JAK-STAT components. Consistently, we found that the 2X STAT-GFP reporter becomes ectopically expressed in later stage cyst cells upon inactivation of E(Pc) in CySC lineage (**Fig. 3-11C-C"**). However, this reporter uses the upstream regulatory sequences from the *Socs36E* gene (Bach et al., 2007), which acts as a repressor of the JAK-STAT signaling (Callus and Mathey-Prevot, 2002; Karsten et al., 2002). This negative feedback regulation of the JAK-STAT signaling might underlie the result that removal of one copy of *stat* gene (using null allele $stat^{06346}$) did not efficiently suppress the *Tj>E(Pc) RNAi* phenotype (data not shown). Furthermore, studies exploring JAK-STAT targets in *Drosophila* optic lobe identified E(Pc) as a target positively regulated by JAK-STAT signaling (Wang et al., 2013), suggesting intricate feedback regulation between E(Pc) and JAK-STAT signaling.

Together, we found that E(Pc) regulates multiple signaling pathways and may act as a master regulator in coordinating somatic and germline lineages in the *Drosophila* adult testis. The ultimate read-out of the changed signaling activity in the absence of E(Pc) depends on the particular targets of E(Pc) in the corresponding signaling pathway and is complicated because E(Pc) has dual roles in either activating or repressing target gene expression. However, this complication of the molecular mechanisms of E(Pc) might be necessary to fine tune the signaling activities which are in accordance with their distinct requirements in promoting stem cell differentiation.

In summary, we demonstrate that an uncharacterized chromatin factor E(Pc) acts in cyst cells which is responsible for germline differentiation and cell fate maintenance. These results emphasize the importance of the microenvironment that germ cells reside in antagonizing

somatic identity and promoting germ cell differentiation. Similar with *Drosophila* testis, many mammalian stem cell niche support more than one stem cell. For example, both hair follicle stem cells (HFSCs) and melanocyte stem cells (MSCs) co-occupy the hair follicle bulge (Nishimura et al., 2002; Oshima et al., 2001). HFSC has been shown to function as a niche for MSC through TGF-β signaling (Tanimura et al., 2011). Similarly, mesenchymal stem cells (MSC) and hematopoietic stem cells (HSC) co-exist in the bone marrow and MSC constitute HSC niche (Mendez-Ferrer et al., 2010). Our findings regarding how two stem cell lineages coordinate during differentiation may apply to other complex niches which support multiple stem cell populations.

MATERIALS AND METHODS

Fly strains and husbandry

Flies were raised under standard yeast/molasses medium at 25°C unless stated otherwise. The following flies were used: *E(Pc)^{1}* (Bloomington Drosophila Stock Center, BL3056), *UAS-E(Pc) dsRNA* (BL28686), *UAS-E(Pc) shRNA* (BL35271), *upd-Gal4* (from D. Harrison, University of Kentucky, Lexington, KY, USA), *Tj-Gal4* (Kyoto stock center, DGRC#104055), *Egfr^{f2}*(BL2768), *yan^{IP}*(BL3101), *E(z)^{731}* (BL24470), *Df[E(z)]* (BL29023), *UAS-yan.ACT* (BL5789), *Vein-lacZ* (BL11749), 2X STAT-GFP (Bach et al., 2007), *Stat92E^{06346}* (from N. Perrimon, Harvard Medical School, Boston, MA, USA), *UAS-Tip60 dsRNA* (BL28563), *UAS-dTip60^{E431Q}, UAS-Tip60* (from Felice Elefant, Drexel University, Philadelphia, Pennsylvania, USA), *Bam-HA* (Eun et al., 2013), *hs-FLP; Act5c.FRT-CD2-FRT.Gal4; UAS-GFP* flies (from Allan Spradling) (Neufeld et al., 1998).

To study the function of E(Pc) in cyst cells, two independent RNAi lines *UAS-E(Pc) dsRNA* and *UAS-E(Pc) shRNA* were crossed with different drivers *upd-Gal4* and *Tj-Gal4* at

25°C, respectively. Newly enclosed progenies were shifted to 29°C and maintained for 8-10 days (D) before dissection. For Tip60 function study, RNAi line *UAS-Tip60 dsRNA* and dominant negative HAT-deficient line *UAS-Tip60^{E431Q}* were crossed with *Tj-Gal4* at 25°C and then adult progenies were shifted to 29°C and maintained for 8-10 D before dissection.

To identify if *E(Pc)* genetic interacts with *Egfr*, *yan*, *E(z)*, *Stat92E*, alleles *Egfr^{f2}*, *yan^{IP}*, *E(z)^{731}*, *Stat92E^{06346}* and deficiency lines *Df [E(z)]* were used. Flies with the following genotypes: *Tj-Gal4/ Egfr^{f2}; UAS-E(Pc) dsRNA/+*, *Tj-Gal4/+; UAS-E(Pc) dsRNA/+* were shifted to 29°C for 3D before analysis. Flies with the following genotypes: *Tj-Gal4/yan^{IP}; UAS-E(Pc) shRNA/+* ,*Tj-Gal4/+; E(z)^{731} / UAS-E(Pc) shRNA*, *Tj-Gal4/+; Df [E(z)]/UAS-E(Pc) shRNA*, *Tj-Gal4/+; UAS-E(Pc) shRNA/ Stat92E^{06346}*, were shifted to 29°C for 5D before dissection.

To study if expression of *E(Pc) cDNA-GFP* in cyst cells is sufficient to rescue *Tj>E(Pc) RNAi* phenotype, flies with the following genotype: *Tj-Gal4/ UAS-E(Pc) cDNA-GFP; UAS-E(Pc) shRNA/+* , *Tj-Gal4/ UAS-E(Pc) cDNA-GFP; UAS-E(Pc) dsRNA/+* were dissected at 5D after shifting from 25°C to 29°C. To test potential defects by overexpression of E(Pc) or Tip60, testes from *Tj-Gal4/ UAS-E(Pc) cDNA-GFP* , *Tj-Gal4/ +;UAS-Tip60 cDNA/+* males 10-11D after shifting from 25°C to 29°C were analyzed.

Generation *E(Pc)* knockdown cyst cell clones

To analyze the function of E(Pc) in individual cyst cells, flies with the following genotype: *hs-FLP; Act5c.FRT-CD2-FRT.Gal4/+;UAS-GFP/UAS-E(Pc) shRNA* were heat shocked at pupal stages for two days with two hours on each day. Enclosed flies were aged for 5-6D after heat shock and used for dissection and immunostaining.

Transgenic fly lines generation

For transgenic fly *UASp-E(Pc) cDNA-GFP*, E(Pc) cDNA was amplified using cDNA prepared from wild-type testis as the template. The 5' half of E(Pc) cDNA was amplified as a KpnI and NotI flanked fragment with E(Pc) F1 and R1 primers. The 3' half of E(Pc) cDNA was amplified as a NotI and XbaI flanked fragment using E(Pc) F2 and R2 primers. These two fragments were then ligated into a pGEM-T-easy vector (Promega) followed by sequencing. To insert the *GFP* sequences at the 3'-end of *E(Pc)* cDNA, a Pml I site was generated right upstream of the stop codon of *E(Pc)* within R2 primer. *GFP* fragment was amplified as a Pml I and Xba I flanked fragment with Primer 5' GFP and 3' GFP, followed by ligation into pGEM-T-E(Pc) 3' half cDNA opened with Pml I and XbaI restriction enzyme digestion. Finally, the 5' half *E(Pc)* cDNA in a KpnI to NotI fragment and the 3' half with GFP in a Not I to Xba I fragment were ligated into pBlueScript vector (Agilent Technologies) cut with Kpn I and Xba I in a 3-way ligation to generate a KpnI and XbaI flanked full-length E(Pc) cDNA tagged with GFP. Then the E(Pc) full cDNA tagged with GFP was cut with Kpn I and Xba I and ligated into UASp vector cut using same two enzymes.

To generate E(Pc) genomic plasmid tagged with GFP, a 21 kb P[acman] BAC clone (CH322-140G22) covering the entire *E(Pc)* genomic region was ordered from BACPAC Resources Center (BPRC). Zra I is one unique enzyme site close to the stop codon of *E(Pc)* genomic region. Pac I is another unique enzyme site within the 3'UTR region of *E(Pc)*. Using primers 3'UTR F and 3'UTR R ended with Asc I and Pac I, an approximate 3 Kb fragment was amplified using the BAC clone as template and ligated into the pGEM-T easy vector. Using primers GFP F and GFP R ended with Zra I and Asc I GFP sequence was amplified. Then, the GFP in a Zra I to Asc I fragment was ligated into pGEM-T 3'UTR vector, cut with AscI and PacI

to generate a GFP-3' UTR fragment flanked by ZraI and PacI. Then GFP-3' UTR cut with ZraI and PacI was ligated into P[acman], opened with ZraI and PacI to generate E(Pc) genomic plasmid tagged with GFP.

Transgenic fly lines were generated by Bestgene Inc (Chino Hills, CA). More than three independent transgenic lines were generated for each transgene.

Primers:

E(Pc) F1: GGGGTACCATGTCCAAGCTGTCGTTCAGAGCCC

E(Pc) R1: ATAAGAATGCGGCCGCTGCCGCCGA

E(Pc) F2: ATAAGAGCGGCCGCCGCTCGTGC

E(Pc) R2: GCTCTAGATCACACGTGTCTGTTGATGGTTGACGTCACAC

5' GFP: ATCACGTGATGGTGAGCAAGGGCGAGGAG

3' GFP: GCTCTAGATTACTTGTACAGCTCGTCCATGCCG

3'UTR F: GGCGCGCCGACGCGGATGGCAGCG

3'UTR R: CCTTAATTAAACATACATACGTATTCTTTTTGTTTTGG

GFP F: GACGTCAACCATCAACAGAATGGTGAGCAAGGGCGAG

GFP R: GGCGCGCCTTACTTGTACAGCTCGTCCATGC

Immunostaining

Testes were dissected in 1X PBS and then fixed in 4% formaldehyde in 1X PBS for 30 min at room temperature (RT). Then testes were washed twice with 20min each time using 1X PBST (0.1% triton) at RT. Testes were incubated with Primary antibodies on a nutator at 4°C overnight. After twice wash with 1X PBST, testes were incubated with secondary antibodies in darkness at RT for 2 hours. After twice wash with 1X PBST, testes were mounted using

Vectashield (Vector H-1200). Primary antibodies used are: Vasa (Rabbit, Santa Cruz, sc-30210), Vasa (Rat, 1:100, developed by Spradling, A. C./ Williams, D. obtained from DSHB), Zfh-1 (Rabbit, 1:5000, from R. Lehmann), Fas III (Mouse, 1:100, DSHB, 7G10), Armadillo (Mouse, 1:200, DSHB, N2 7A1), Eya (Mouse, 1:25, DSHB, 10H6), Yan (Mouse, 1:25 after pre-absorption against *Drosophila* embryos, DSHB, 8B12H9), GFP (Chicken, 1:1000, Abcam, ab13970), dpERK (Rabbit, 1:100, Cell signaling, #4370), HA (Rat, 1:50, Roche, 3F10), β-Galactosidase(Mouse, 1:200, Sigma, G4644). For dpERK staining, testes were dissected in 10 mM Tris-Cl pH 6.8, 180 mM KCl, 50 mM NaF, 1 mM Na3VO4, 10 mM b-glycerophosphate as described before (Kiger et al., 2000). Secondary antibodies were all Alexa Fluor series (1:200, Molecular Probes). Images were taken with Zeiss LSM 510 META or LSM 700. Images were processed using Adobe Photoshop. EdU incorporation was performed with Click-iT EdU Alexa Fluor 488 imaging kit (Invitrogen C10083). Dissected testes were incubated with EdU solution for 30min, followed by fixation and immunostaining as described.

Quantification of Vein Intensity and data analysis

To compare Vein intensity between the *Tj-Gal4* control and *Tj>E(Pc) shRNA* testes, H2Av-mRFP (BL34498) transgene was used as a marker to distinguish the two genotyped fly testes. Testes dissected from *Tj-Gal4/H2Av-mRFP; Vein-LacZ/+* males were compared with *Tj-Gal4/+; Vein-LacZ/UAS-E(Pc) shRNA* testes, which were immunostained together and imaged using the same parameters. Control testes could be identified based on the H2Av-mRFP marker. Vein-LacZ fluorescence intensity was measured for each Z stack across the entire nucleus using Image J software and summed up. Data were analyzed and presented using GraphPad Prism software.

Chromatin immunoprecipitation sequencing (ChIP-seq) and data analysis

Flies with following genotype: *Tj-Gal4/ UAS-E(Pc) cDNA-GFP; UAS-E(Pc)shRNA/+* were collected as newly eclosed males and aged for 5D at 29°C after the shift from 25°C. Approximately 2,000 pairs of testis were dissected and grouped into two batches which were used as two replicates for ChIP experiments, which were performed using ChIP-IT high sensitivity kit (#53040, Active motif) following the manufacturer's instruction. Sonication of fixed testes was performed using Bioruptor sonicator (UCD-200, diagenode) using the following setting: 0.5min ON, 1min OFF repetitively for a total of 25min. The size of DNA associated with sonicated chromatin was checked which was approximate 400-500 bp.

Libraries were generated using reagents provided in the Illumina®TruSeq® ChIP Sample Preparation Kit (IP-202-1012). The Illumina compatible libraries were sequenced with Mi-seq desktop sequencer (Mi-Seq, Illumina). Then 75 bp pair-end read sequencing was performed. FASTQ raw data files were filtered with quality control software Fastqc (www.bioinformatics.babraham.ac.uk/projects/fastqc/). BOWTIE program [version 0.12.7, (Langmead et al., 2009)] was utilized to align reads to *Drosophila* genome (dm3), with the running parameters (bowtie -p 8 -t -a --phred33 -quals -n 2 -e 70 -l 48 -m 1 --best --strata). Pair-end reads were treated as separate single reads. At each chromosome position, only one read was retained to get a non-redundant read count data. SAM formatted alignment files will be uploaded onto NIH GEO database upon paper acceptance. Enrichment of reads across the genome was analyzed by MACS2 (Zhang et al., 2008) for peak calling. The peak calling was performed with paired experiment (GFP ChIP) and control genome input under default parameter settings. UCSC genome browser customized visualization tools were also applied in the analysis (Kent et

al., 2002). SAMtools (Li et al., 2009) software suite was utilized to convert between related read formats. Go term analysis was performed using Gorilla (Eden et al., 2007; Eden et al., 2009).

RNA-seq and data analysis

One pair of *Tj-Gal4* or *Tj-Gal4/+; UAS-E(Pc) shRNA/+* testes were dissected in PBS, respectively as one replicate. Two replicates were generated for each genotype. Total RNA was purified following the manufacturer's instruction of PicoPure RNA isolation kit (KIT0204, Life technologies). Then both libraries were generated using reagents provided in Illumina®TruSeq® RNA Sample Preparation Kit (RS-122-2001). The Illumina compatible libraries were sequenced with Illumina Hiseq2500 sequencer in the high-throughput sequencing core facility at Johns Hopkins University Bayview with 50 bp single-end reads.

For the alignment to fly genome and gene mapping, sequencing reads were examined by fastqc quality control software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The reads which passed quality filter were mapped back to *Drosophila* genome (dm3) (FlyBase dmel_r5.43, as of Jan 2012, ftp://ftp.flybase.net/releases/FB2012_01/dmel_r5.43/). Bowtie aligner (version 0.12.7) (Langmead et al., 2009) was utilized with the following configuration (-a --phred33-quals -n 2 -e 70 -l 28 -m 1 --best –strata) which allows two mismatches and only one alignment site. We then assigned each read into gene regions. The annotation for protein coding genes was retrieved from FlyBase database (as of Jan 2012, ftp://ftp.flybase.net/releases/FB2012_01/dmel_r5.43/). The exons from different alternative splicing isoforms were merged to find the maximum genome coverage regions per gene. When a read is mapped to a region with more than one gene, i.e., one merged exon region overlaps with a non-coding gene, the count is split as equal possibilities into these two genes with the half count

for each. A matrix file with the number of reads assigned to each gene per sample was prepared for the following data analysis.

To identify differentially expressed genes, we utilized the edgeR software package (Robinson et al., 2010) in *R* to find the normalization factors for each sample with various sizes [by the TMM (Trimmed Mean of M value) and upper quantile normalization methods]. The edgeR method models short reads into negative binomial distribution and estimates the biological replicate variance (dispersion). Tag-wise dispersion estimation was performed in "*Tj-Gal4", "Tj-Gal4/+; E(Pc) shRNA/+"* two groupings of read count profiles. We introduced quantity term "corrected RPKM (cRPKM)" by the formula: pseudo.alt * 1e+09 / (length of merged transcripts)/ (common.lib.size). The common.lib.size was calculated from the calcNormFactors function of edgeR, which performs TMM and upper quantile normalization methods and set a reference library. The pseudo.alt contains read counts after normalization across the input multiple profiles. The pseudo.alt was calculated by edgeR using quantile normalization and maximum likelihood method. The pseudo.alt contains pseudo read counts after correcting the library size and composition differences.

After cRPKM calculation, gene expression levels per sample were pair-wisely compared with spearman correlation (correlation coefficient rho). A pair-wise inter-profile distance was defined as (1-rho) and set up a distance matrix. A dimension reduction method, multidimensional scaling in *R* (http://stat.ethz.ch/R-manual/R-devel/library/stats/html/cmdscale.html), was utilized to visualize the global similarity relationship of 4 samples.

FIGURES

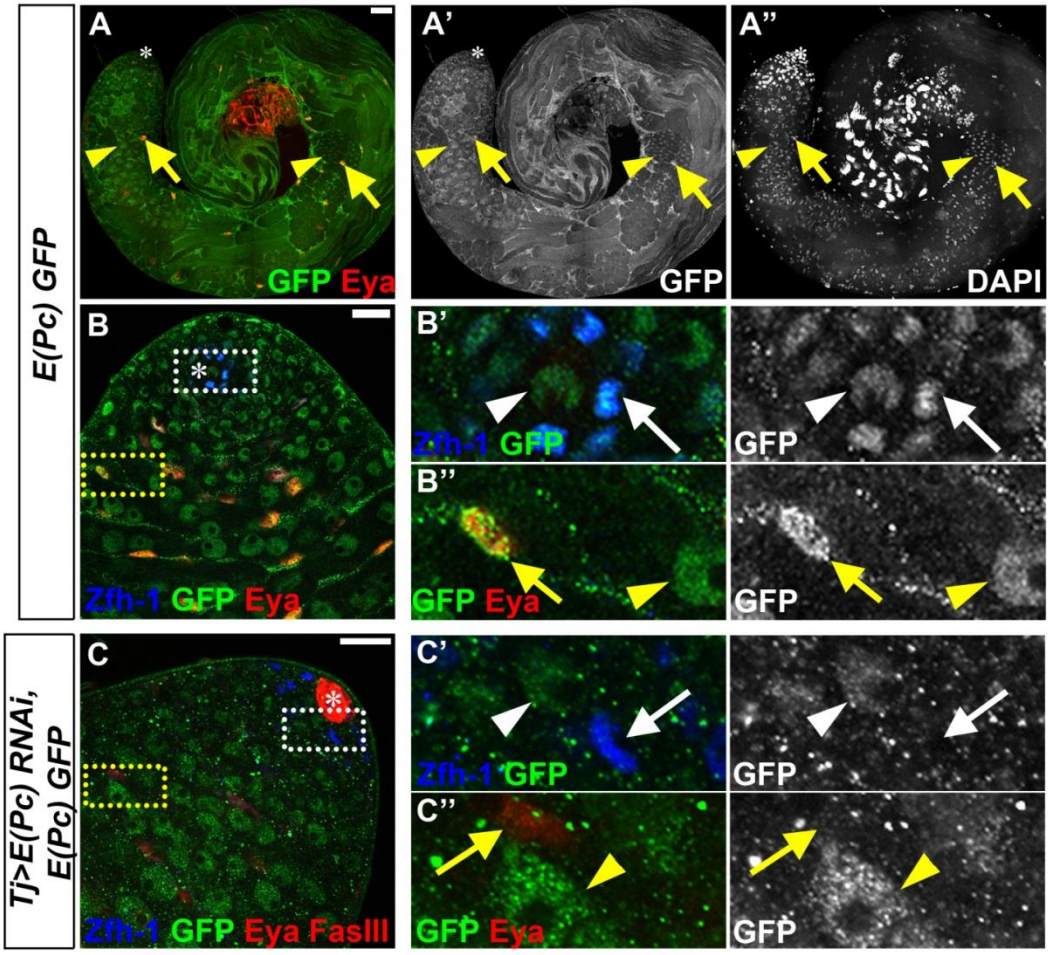


Figure 3-1: Expression pattern of E(Pc)-GFP protein in *Drosophila* adult testes. (**A-A"**) GFP signal was detected in nuclei of germ cells (yellow arrowheads) and Eya-positive late-stage cyst cells (yellow arrows) throughout testes. Scale bar: 50μm. (**B-B"**) At the apical tip of testis, GFP signal is enriched in nuclei of germ cells (white arrowhead in **B'** and yellow arrowhead in **B"**), early cyst cells (Zfh-1-positive cell, labeled by white arrow in **B'**) and later cyst cells (Eya-positive cell, labeled by yellow arrow in **B"**). (**C-C"**) In *Tj>E(Pc) RNAi, E(Pc) GFP* testes, no GFP signal was detectable in early cyst cells (Zfh-1-positive cell, labeled by the white arrow in **C'**) and later cyst cells (Eya-positive cell, labeled by the yellow arrow in **C"**). GFP signal in

germ cells was not affected (white arrowhead in **C'** and yellow arrowhead in **C"**). Asterisk: hub. Scale bar: 20μm.

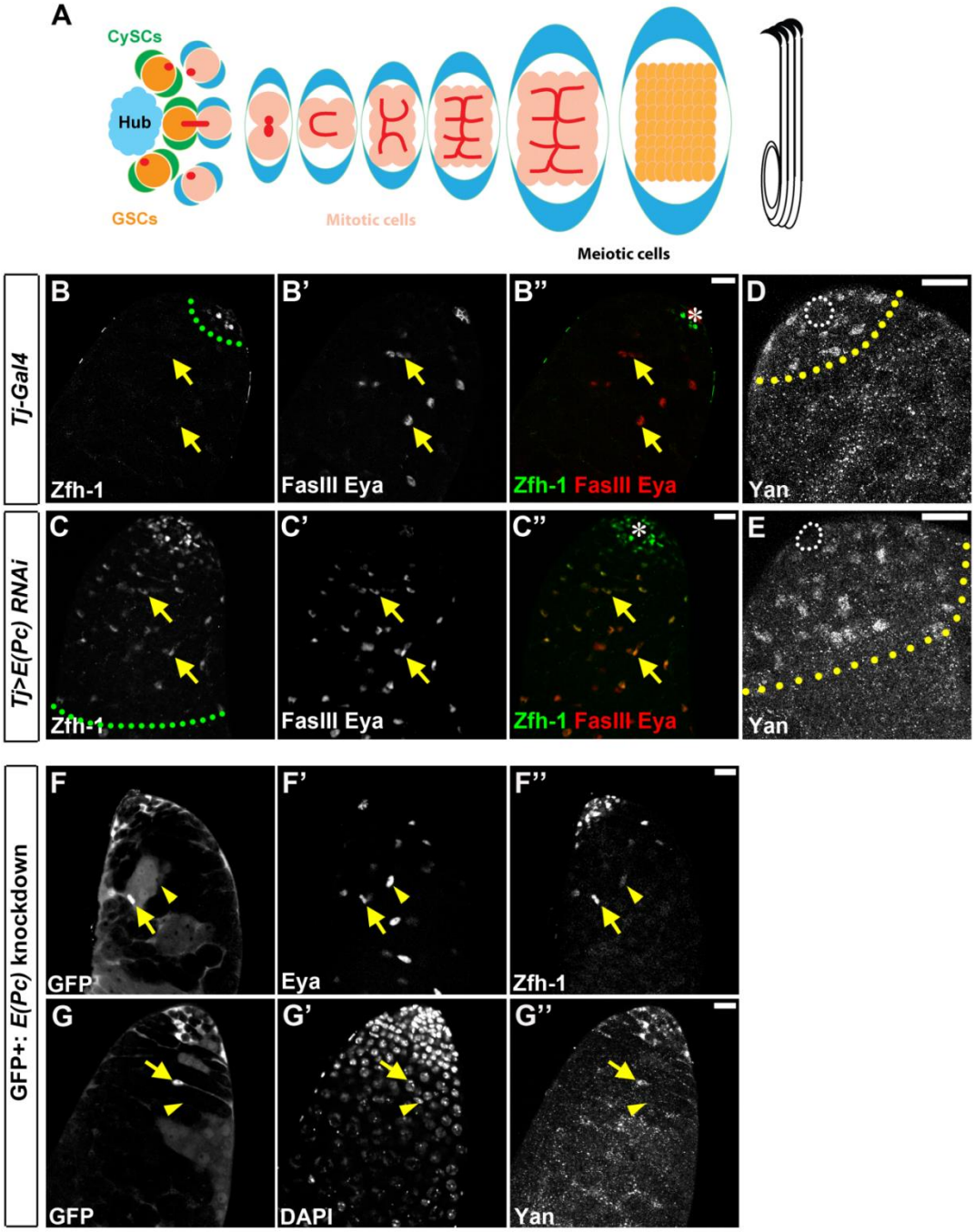


Figure 3-2: E(Pc) downregulates CySC-enriched transcription factors to promote cyst cell differentiation. (A) A schematic diagram of *Drosophila* adult testes. CySCs: cyst stem cells; GSCs: germline stem cells. **(B-B")** In *Tj-Gal4* control testes, Zfh-1-positive early cyst cell zone (green dashed line) is separable spatially from the region with later stage cyst cells labeled by Eya (yellow arrows). **(C-C")** In *Tj>E(Pc) RNAi* testes, Zfh-1-positive cell zone expands (green dashed line) with some cells co-expressing Eya (yellow arrows). **(D)** In *Tj-Gal4* control testes, another marker Yan is highly enriched in CySCs and early cyst cells (yellow dashed line), a few cell-diameter away from the hub region (white outline). **(E)** In *Tj>E(Pc) RNAi* testes, Yan-positive cell zone (yellow dashed line) expands and is further away from the hub (white outline). **(F-G")** GFP-positive cells represent cells with *E(Pc)* knockdown. Compared with the GFP-negative and Eya-positive cyst cell (yellow arrowhead in **F-F"**), the neighboring GFP-positive and Eya-positive cyst cell (yellow arrow in **F-F"**) has a higher Zfh-1 signal. Similarly, compared with GFP-negative and DAPI-positive cyst cell (yellow arrowhead in **G-G"**), the neighboring GFP-positive and DAPI-positive cyst cell (yellow arrow in **G-G"**) has higher Yan signal. Asterisk: hub. Scale bar: 20μm.

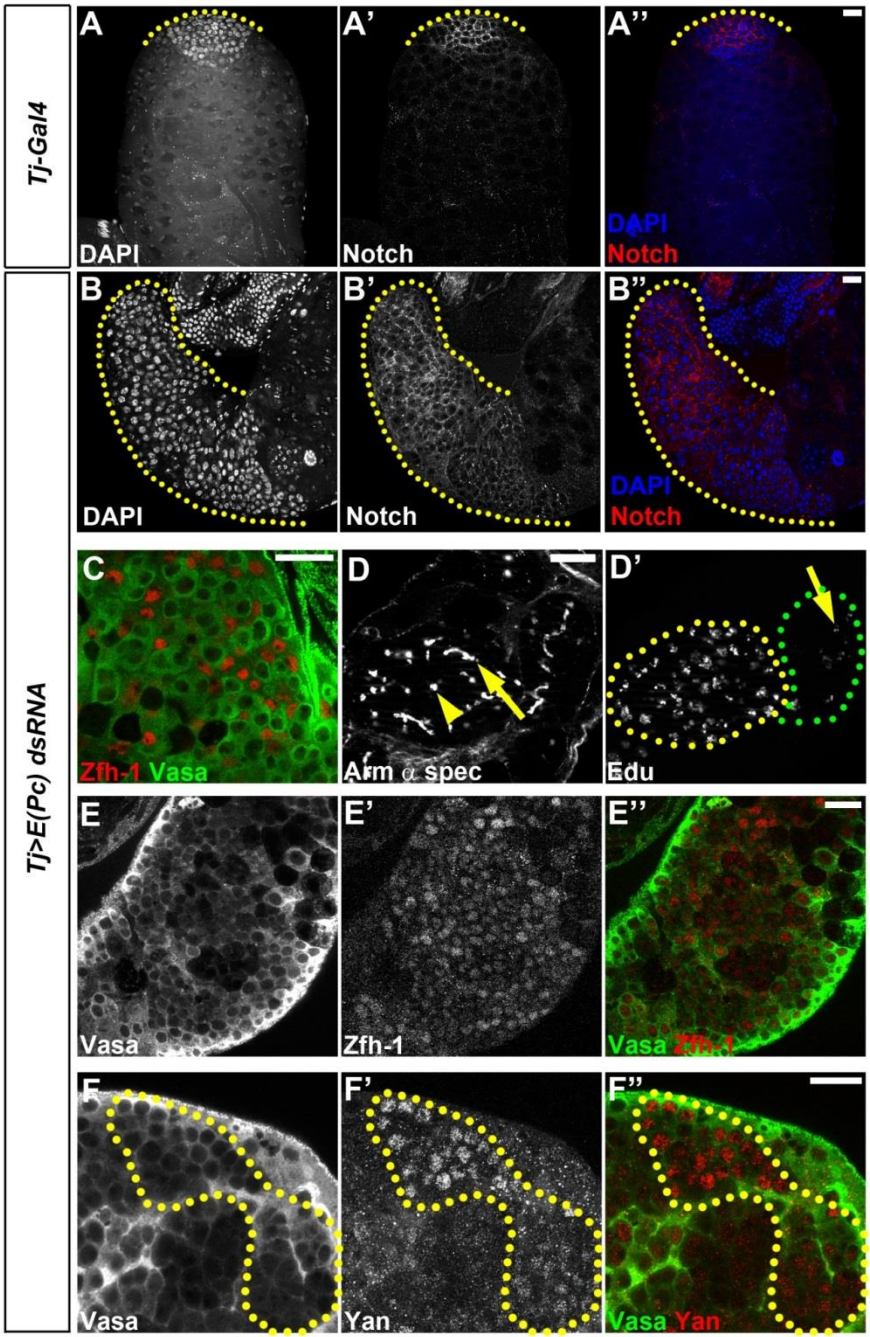


Figure 3-3: E(Pc) acts in cyst cells to promote germ cell differentiation and maintain germline identity. (A-A") In *Tj-Gal4* control testes, DAPI bright region (yellow dashed line in **A**), and Notch positive cells (yellow dashed line in **A'**) represent GSCs and early-stage germ cells. **(B-B")** Elongated DAPI bright region (yellow dashed line in **B**) and Notch-positive cell zone (yellow dashed line in **B'**) in *Tj>E(Pc) dsRNA* testes. (**C**) Immunostaining with germ cell

marker Vasa (Green) and early cyst cell marker Zfh-1 in *Tj>E(Pc) dsRNA* testes: GSC- and GB-like germ cells intermingle with Zfh-1 positive cells. (**D-D'**) Immunostaining with antibodies against Armadillo and α–spectrin **(D)** in *Tj>E(Pc) dsRNA* testes show overproliferative spermatogonial tumor cells interconnected with both round spectrosome (yellow arrowhead) and branched fusome (yellow arrow). EdU labeling in *Tj>E(Pc) dsRNA* testes (**D'**) show only a subset of over-proliferating germ cells within one cyst are EdU-positive (yellow arrow). (**E-F"**) In *Tj>E(Pc) dsRNA* testes, Vasa-positive germ cells (**E**, **F**, green in **E"** and **F"**) are also labeled with CySCs-enriched marker Zfh-1 (**E'**, red in **E"**) and Yan (**F'**, red in **F"**). Scale bar: 20μm.

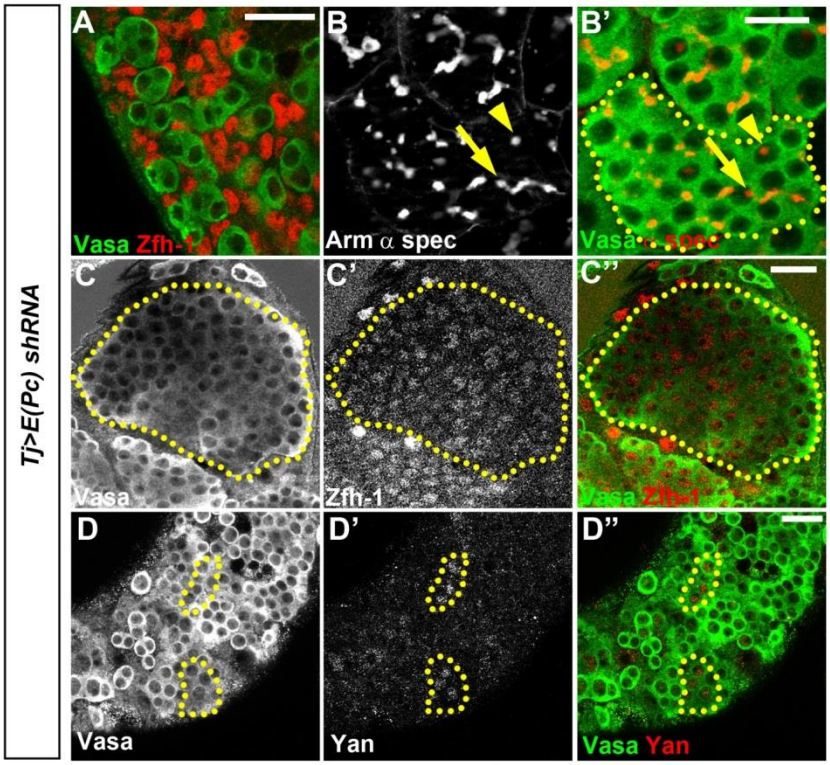


Figure 3-4: Knockdown of *E(Pc)* in cyst cells using a different short hairpin (sh) RNA also leads to germ cell overproliferation and ectopic expression of cyst cell markers.

Immunostaining using the germ cell marker Vasa (**C** and **D**, green in **A**, **B'**, **D"**), an early cyst cell marker Zfh-1 (**C'**, red in **A**, **C"**), an early cyst cell marker Yan (**D'**, red in **D"**), Armadillo

and α spectrin (**B**, red in **B'**) in *Tj>E(Pc) shRNA* testes. (**B-B'**) Overproliferating germ cells within one cyst (yellow dashed line based on Arm staining) have both round spectrosome (yellow arrowhead) and branched fusome (yellow arrow). Scale bar: 20μm.

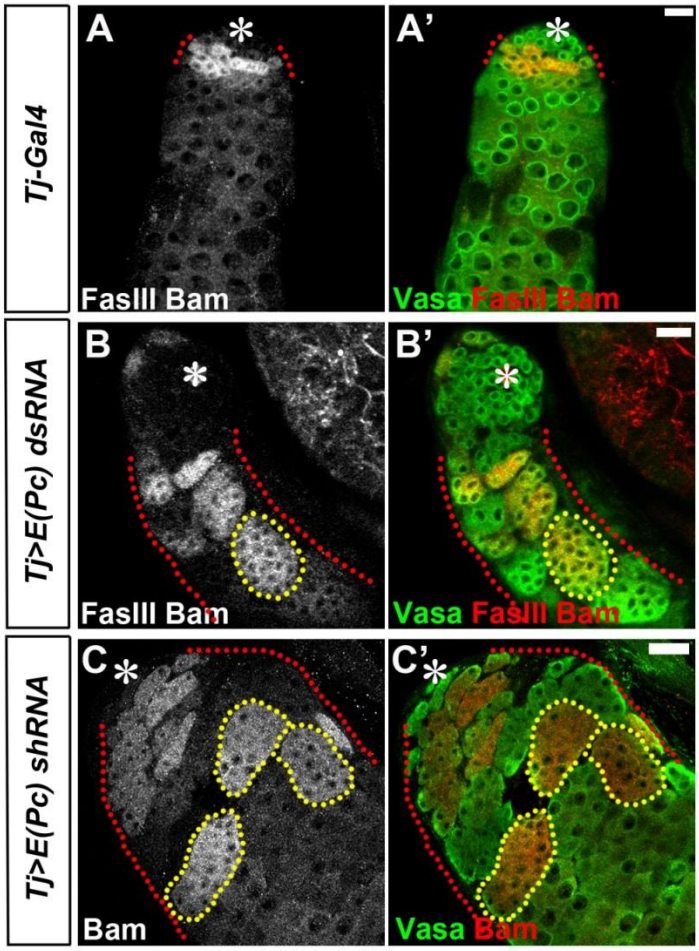


Figure 3-5: Overproliferating germ cells in *Tj>E(Pc) RNAi* testes at the transit-amplifying stage are Bam-positive. (**A-A'**) In *Bam-HA, Tj-Gal4* control testes, immunostaining with anti-HA (red) and anti-Vasa (green) showed Bam expression in 4- to 16- spermatogonial cells (red dashed line). In *Bam-HA, Tj>E(Pc) dsRNA* testes (**B-B'**) and *bam-HA, Tj>E(Pc) shRNA* testes (**C-C'**): Bam is detectable in overproliferating spermatogonial tumor cells (red

dashed line labeled overproliferative cell zone and the yellow dashed line labeled individual spermatogonial tumor cysts). Asterisk: hub. Scale bar: 20μm.

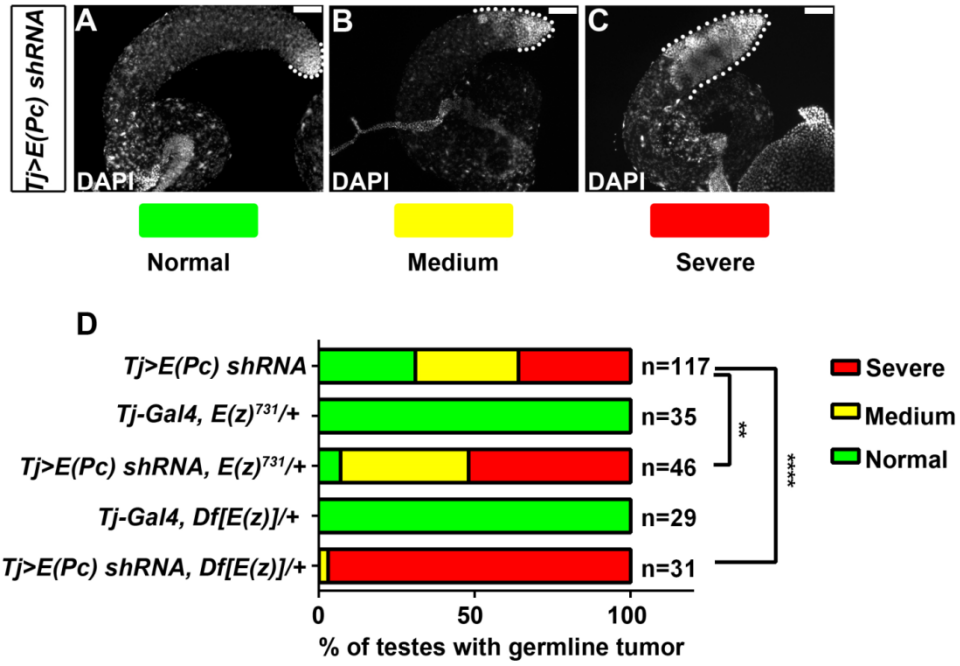


Figure 3-6: Reduction in E(z) significantly enhances the tumor phenotype in *Tj>E(Pc) shRNA* testes. (**A-C**) In *Tj>E(Pc) shRNA* testes, *E(Pc)* knockdown in cyst cells leads to both somatic and germline tumors shown as the expansion of DAPI bright region (white dashed line). Scale bar: 100μm. (**D**) Quantification of the penetrance and severity of the tumor phenotype at different genetic backgrounds. ***P*<0.01, *****P*<0.0001, chi-square test.

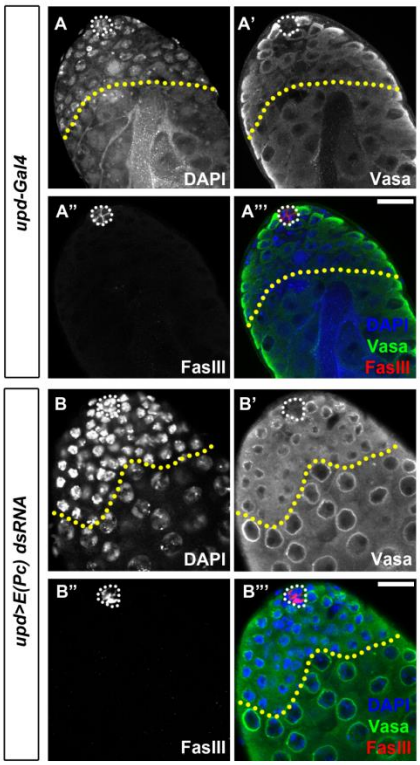


Figure 3-7: Knockdown of *E(Pc)* in hub cells does not lead to any detectable defects. (**A-A'"**) In *upd-Gal4* control testes, transit-amplifying stage germ cells (yellow dashed line) with DAPI bright nuclei localize at the apical tip of the testis. (**B-B'"**) In *upd>E(Pc) dsRNA* testes, no expansion of DAPI bright region was observed as in *Tj>E(Pc) RNAi* testes. White outline: hub region. Scale bar: 20μm.

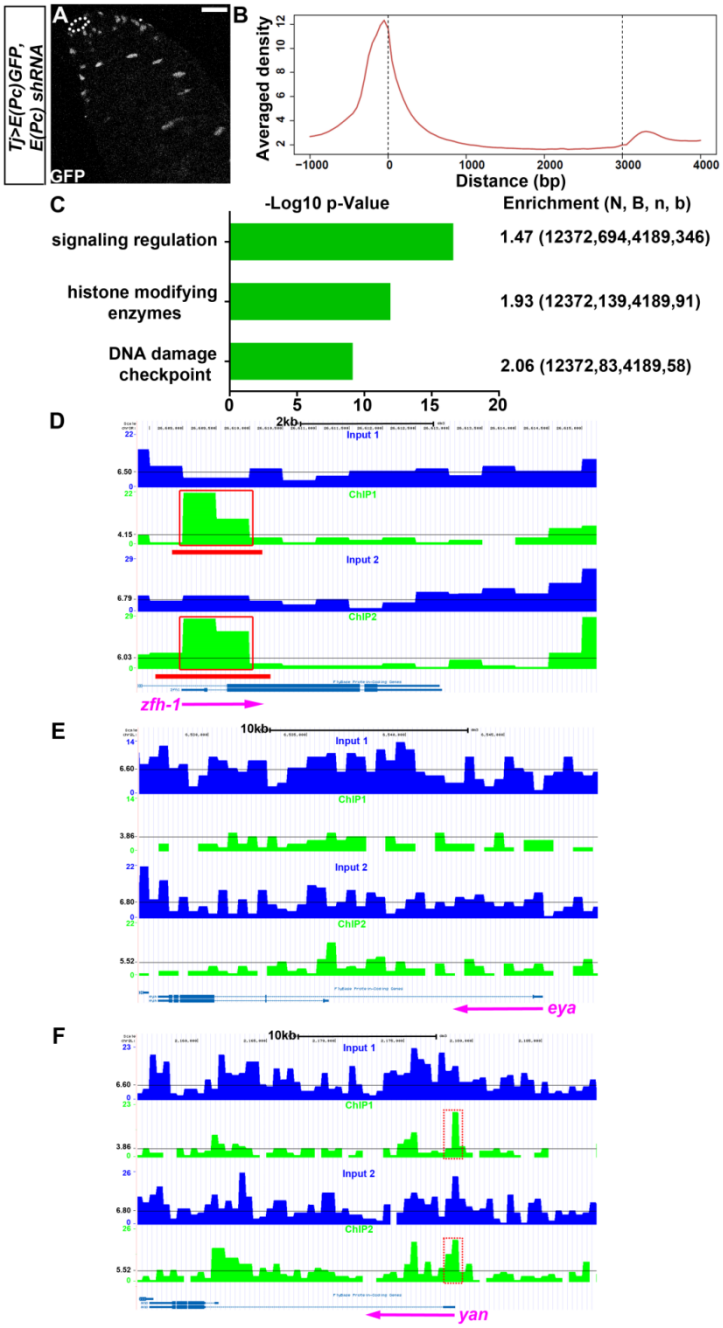


Figure 3-8: E(Pc) directly regulates multiple signaling pathway components and the CySC self-renewal factor Zfh-1. (**A**) In *Tj>E(Pc)cDNA GFP, E(Pc) shRNA* testes, GFP is only detectable in CySC lineage. White dotted line: hub. Scale bar: 20μm. (**B**) ChIP-seq was performed with the GFP antibody using *Tj>E(Pc)GFP, E(Pc) shRNA* testes. Two independent ChIP experiments were performed. Average E(Pc) enrichment signal profile of 4698 genes over

a -1-kb to +4-kb region with respect to the transcription start sites (TSSs). (**C**) GO term enrichment test to identify significant categories with distinct biological functions among E(Pc)-binding genes. Enrichment (N, B, n, b): N- total number of genes, B- total number of genes associated with a specific GO term, n- the number of all E(Pc) target genes, b- the number of E (Pc) target genes with this specific GO term. The scores mean overall enrichment of genes within annotated GO term. -Log 10 P-value annotates the significance of genes enrichment within this specific GO term. (**D**) A genome browser snapshot of the *zfh-1* gene region. Compared with the input control, anti-GFP immunoprecipitated chromatin from cyst cells show enrichment around *zfh-1* promoter region, indicated by red lines below read density map and red box. The black line indicates average read density of chromosome 3R. **(E)** A genome browser snapshot of the *eya* gene region. No enrichment around promoter region is observed. The black line indicates average read density of chromosome 2L. **(F)** A genome browser snapshot of the *yan (*also known as *aop)* gene region. Local enrichment (labeled by a red box with dashed line) could be detected around promoter region, although the enrichment did not pass the threshold using peak MACS2 calling algorithm. The black line indicates average read density of chromosome 2L.

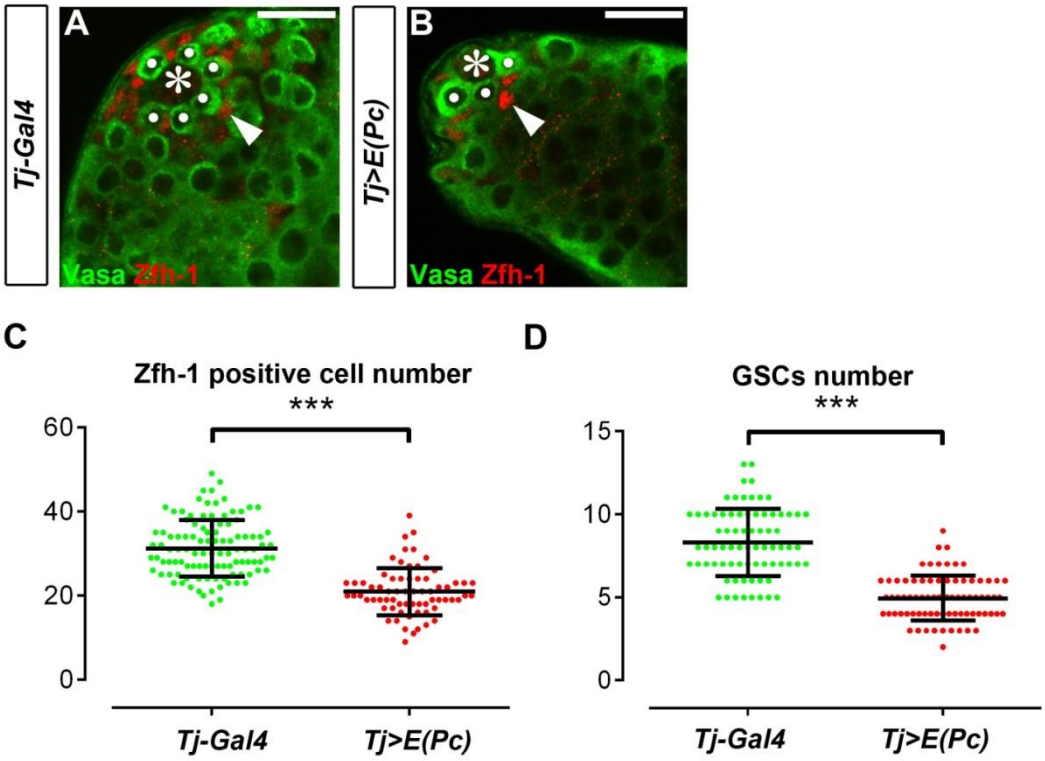


Figure 3-9: Overexpression of E(Pc) in cyst cells causes defects in the maintenance of Zfh-1-positive early stage cyst cells (including CySCs) and GSCs. (**A-B**) Immunostaining using Vasa (germ cell marker) and Zfh-1 (early cyst cell marker) in *Tj-Gal4* control testes (**A**) and *Tj>E(Pc) cDNA-GFP* testes (**B**). GSCs labeled by white dots and Zfh-1 positive cells by a white arrowhead. Asterisk: hub. Scale bar: 20μm. (**C**) Quantification of Zfh-1-positive cells. *Tj-Gal4*: 31.28 ± 6.69 (Mean ± SD, N=105), *Tj>E(Pc)cDNA-GFP*: 20.97 ± 5.62 (N=68). (**D**) Quantification of GSCs. *Tj-Gal4*: 8.31 ± 2.04 (N=75), *Tj>E(Pc) cDNA-GFP*: 4.95 ± 1.35 (N=78). *** P<0.001. Two-tailed t test.

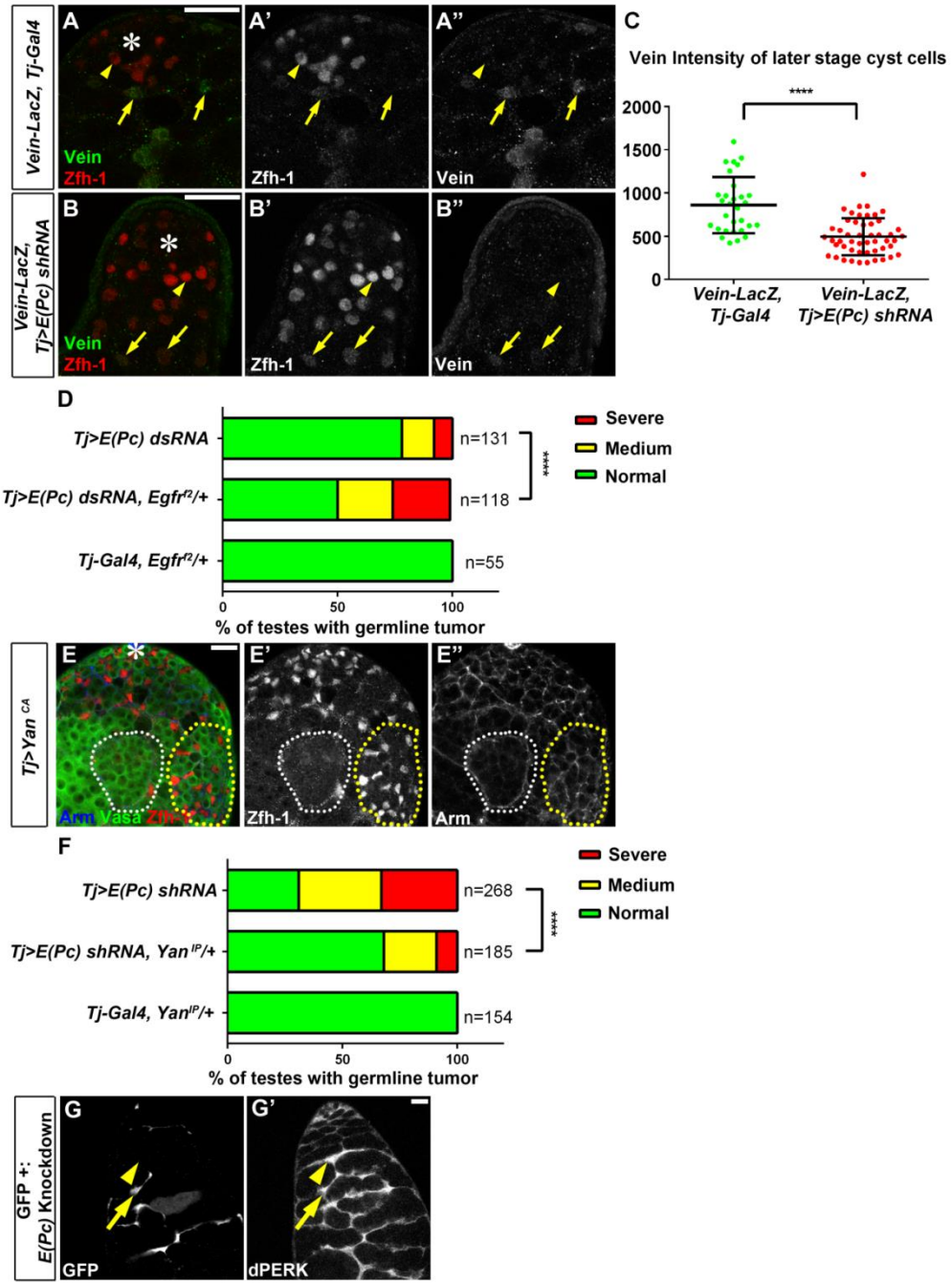


Figure 3-10: E(Pc) acts in synergy with EGF signaling pathway to promote cellular differentiation in both CySC and GSC lineages. (**A-A"**) In *Vein-LacZ, Tj-Gal4* control testes, Vein is not expressed in Zfh-1 positive CySCs (yellow arrowhead) but becomes detectable in differentiated cyst cells (yellow arrows). (**B-B"**) In *Vein-LacZ, Tj> E(Pc) shRNA* testes, Vein is not expressed in CySCs (yellow arrowhead), almost undetectable in later stage cells (yellow

arrows) several cell diameters away from the hub (Asterisk). (**C**) Quantification of Vein expression in later stage cyst cells (labeled by yellow arrows in **A"** and **B"**). ****P<0.0001. Two-tailed t test. (**D**) Quantification of the percentage of testes with expanded DAPI bright region (severe, medium and normal, refer to Fig. 3-6A-C) in *Tj>E(Pc) dsRNA*, *Tj>E(Pc) dsRNA, $Egfr^{f2}$/+,* and *Tj-Gal4, $Egfr^{f2}$/+* testes. Removing one copy of *Egfr* using loss-of-function $Egfr^{f2}$ allele enhanced the phenotype. ****P<0.0001, chi-square test. (**E-E"**) Ectopic expression of consistent active (CA) Yan in cyst cells caused overpopulation of Zfh-1 cells (red in **E**, **E'**) accompanying GSC- and GB-like tumor (yellow dashed line) and spermatogonial tumor (white dashed line) within spermatogonial cyst shown by anti-Arm immunostaining (**E"**). (**F**) Quantification of the percentage of testes with expanded DAPI bright region in *Tj>E(Pc) shRNA*, *Tj>E(Pc) shRNA, Yan^{IP}/+,* and *Tj-Gal4, Yan^{IP}/+* testes, using the same criterion as in (**D**). Removing one copy of *yan* using a null allele yan^{IP} suppressed the phenotype. ****P<0.0001, chi-square test. **(G-G')** Level and localization of phosphorylated ERK (dpERK) had no detectable difference in *E(Pc)* knockdown cyst cells (GFP-positive, yellow arrow), compared with control cyst cells with normal E(Pc) (GFP- negative, yellow arrowhead). Asterisk: hub. Scale bar: 20μm.

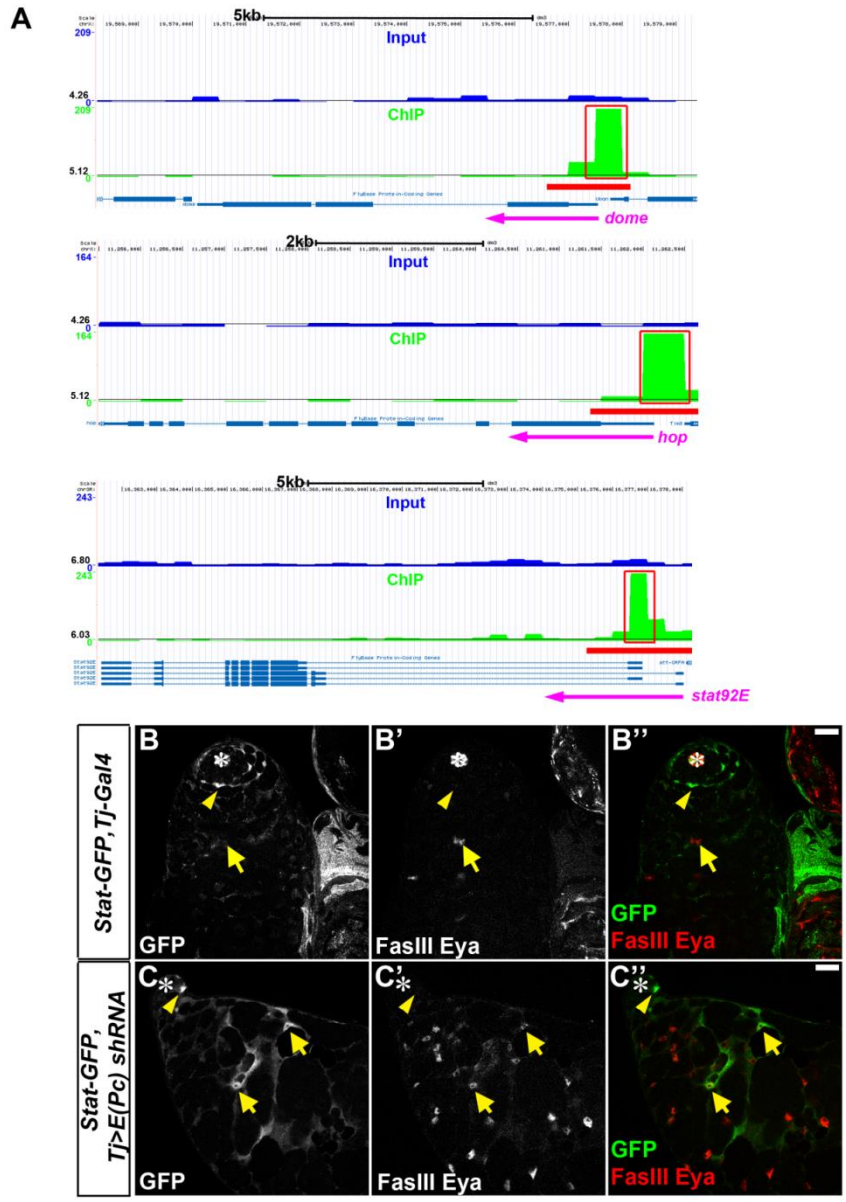


Figure 3-11: E(Pc) represses JAK-STAT signaling to promote cyst cell differentiation. (**A**) Genome browser snapshots of E(Pc) enrichment at *domeless (dome), hopscotch (hop), stat92E* gene loci. One replicate of ChIP experiment is shown here and the other replicate shows a similar pattern. Peak calling is labeled by red lines below read density map and also red box. The black line indicates average read density of relative chromosome. (**B-B"**) In *Stat-GFP, Tj-Gal4*

control testes, GFP signal is enriched in CySCs which are one cell diameter away from the hub (asterisk). GFP-positive cells (yellow arrowhead) are separable from Eya-positive later stage cyst cells (yellow arrow). (**C-C"**) In *Stat-GFP, Tj>E(Pc) shRNA* testes, GFP-positive cells expand from apical CySCs (yellow arrowhead) to Eya-positive cells (yellow arrows). Asterisk: hub. Scale bar: 20μm.

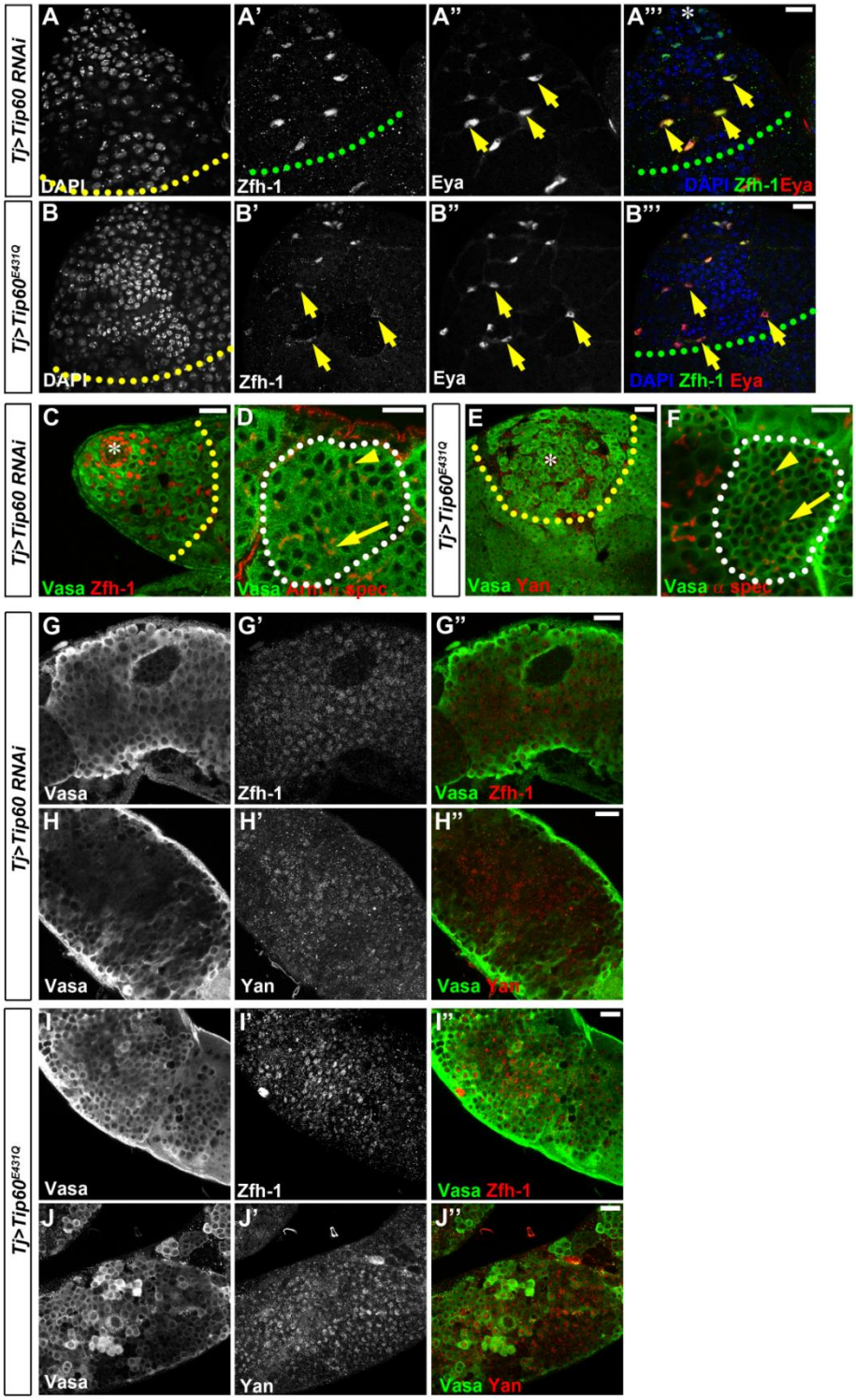


Figure 3-12: E(Pc) acts in synergy with Tip60 whose function in cyst cells depend on its histone acetyltransferase activity. (**A-A'''**) *Tj>Tip60 RNAi* testes show expansion of cells with

DAPI bright nuclei (yellow dashed line in **A**) and Zfh-1-positive cyst cells (green dashed line in **A'** and **A"'**). Now Zfh-1 co-expresses with late-stage cyst marker Eya (yellow arrows). (**B-B"'**) Tip60 HAT-deficient dominant active form (*Tip60*^{E431Q}) expressed using *Tj-Gal4*. *Tj>Tip60*^{E431Q} testes show expansion of cells with DAPI bright nuclei (yellow dashed line in **B**), including cells co-expressing Zfh-1 and Eya (yellow arrows in **B'-B"'**). (**C**) In *Tj>Tip60 RNAi* testes, GSC- and GB-like cells are intermingled with Zfh-1-positive cells (cells from the hub region to yellow dashed line). (**D**) Spermatogonial tumor with more than 16 germ cells (Vasa-positive) within one cyst (white dashed line) interconnected by both branched fusome (yellow arrow) and round spectrosome (yellow arrowhead) in *Tj>Tip60 RNAi* testes. (**E-F**) Similar GSC- and GB-like tumor (cells from hub region to yellow dashed line in **E**) and spermatogonial tumor (white dashed line in **F**) are also detected in *Tj>Tip60*^{E431Q} testes. (**G-H"**) In *Tj>Tip60 RNAi* testes, Vasa-positive cells (**G**, **G", H**, **H"**) also have expression of CySC-enriched marker Zfh-1 (**G'**, **G"**) and Yan **(H'**, **H")**. (**I-J"**) In *Tj>Tip60*^{E431Q} testes, Vasa-positive cells (**I**, **I"**, **J**, **J"**) also have expression of Zfh-1 (**I', I"**) and Yan (**J'**, **J"**). Asterisk: hub. Scale bar: 20μm.

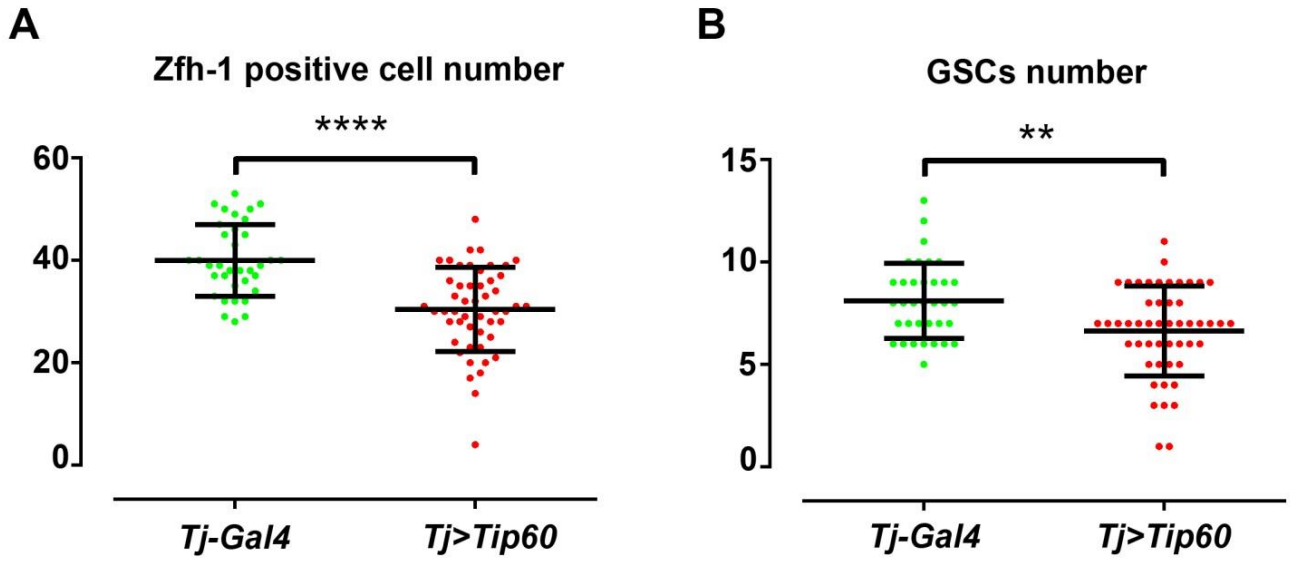


Figure 3-13: Overexpression of Tip60 in cyst cells causes defects in the maintenance of Zfh-1-positive early stage cyst cells and GSCs. (**A**) Quantification of Zfh-1-positive cells. *Tj-Gal4* control testes: 40±6.96 (N=35), *Tj>Tip60 cDNA* testes: 30.42±8.24 (N=50). ****P<0.0001, Two-tailed t test. (**B**) Quantification of GSCs. *Tj-Gal4* control testes: 8.11±1.84 (N=35), *Tj>Tip60 cDNA* testes: 6.64±2.18 (N=50). **P<0.01, Two-tailed t test.

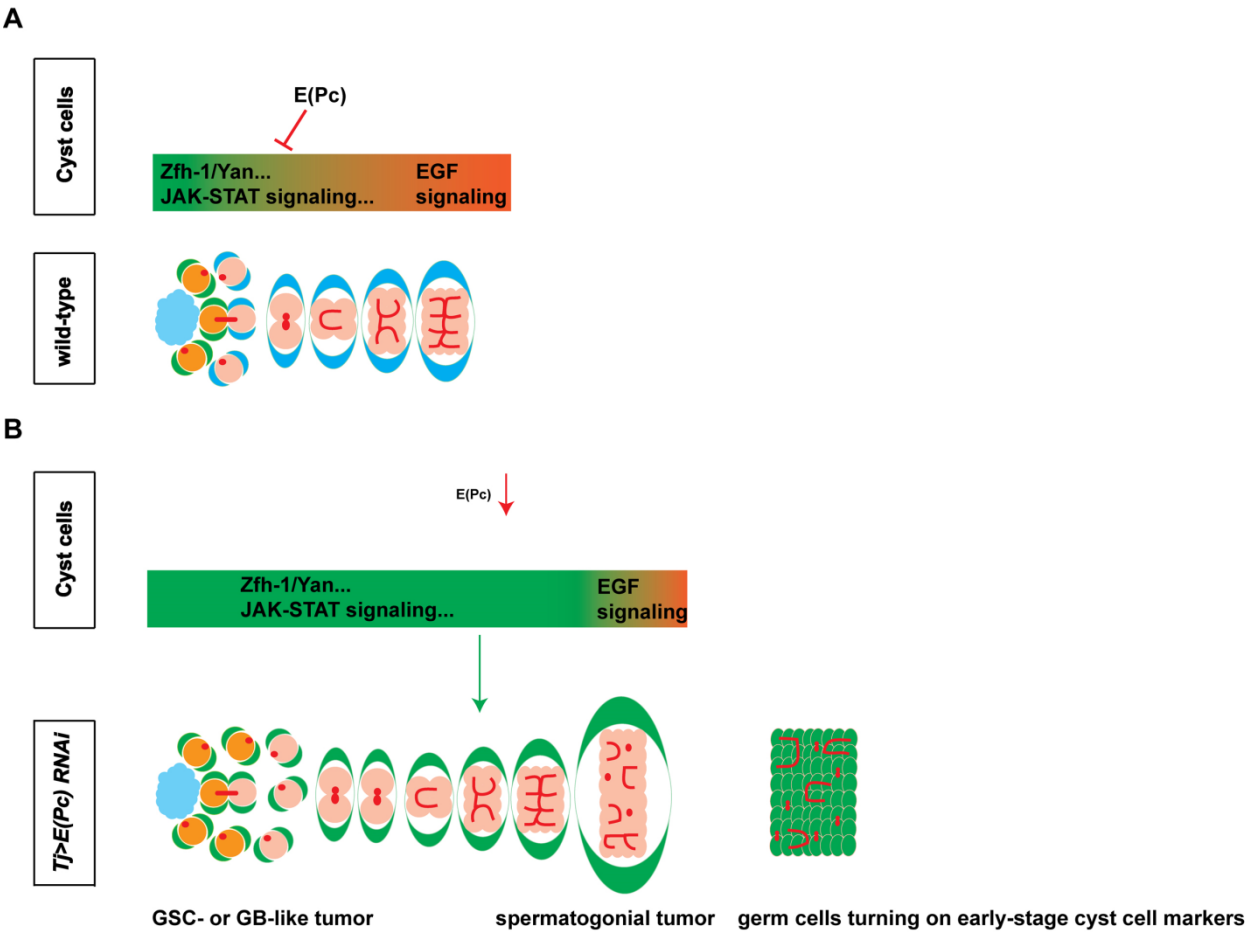
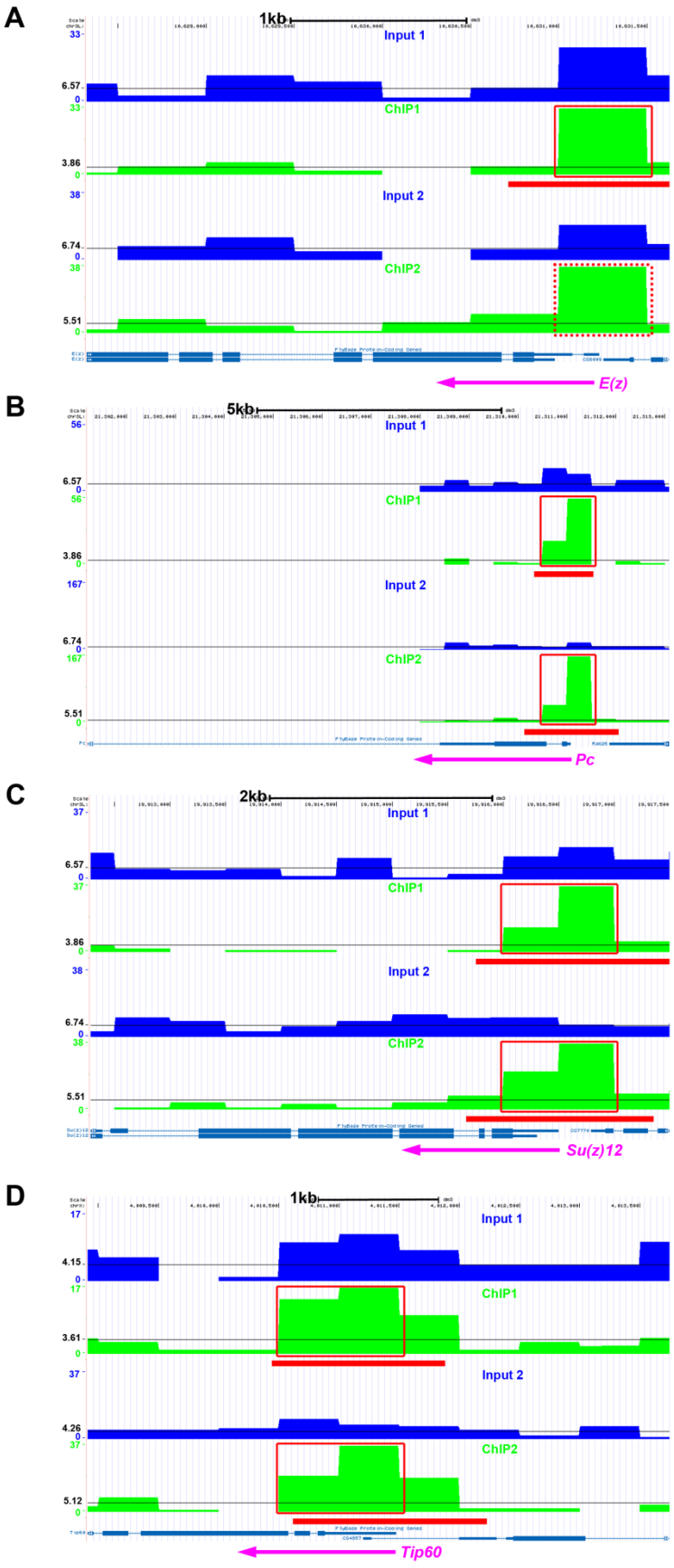


Figure 3-14: A model to describe both the cell-autonomous functions of E(Pc) in CySC lineage and the non-cell-autonomous roles in regulating germ cell differentiation and maintaining germline identity. (**A**) In cyst cells of wild-type testes, E(Pc) represses CySC-enriched factors (e.g. Zfh-1, Yan) and the JAK-STAT signaling pathway to promote cyst cell differentiation cell-autonomously. E(Pc) also acts in synergy with the EGF signaling pathway.

Under this condition, both cyst cell and germ cell differentiate properly. (**B**) When *E(Pc)* is knocked down in cyst cells, ectopic expression of Zfh-1 and Yan, as well as hyperactivation of the JAK-STAT signaling but compromised EGF signaling activity lead to germ cell tumors including both GSC- or GB-like tumor and spermatogonial tumor. In addition, the overproliferative germ cells turn on early-stage cyst cell markers.


A
1kb
Input 1
ChIP1
Input 2
ChIP2
E(z)
B
5kb
Input 1
ChIP1
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ChIP2
Pc
C
2kb
Input 1
ChIP1
Input 2
ChIP2
Su(z)12
D
1kb
Input 1
ChIP1
Input 2
ChIP2
Tip60

Figure 3-15: E(Pc) is enriched at the promoter region of *E(z), Pc, Su(z)12* and *Tip60* genomic loci. Genome browser snapshots of *E(z)* (**A**), *Pc* (**B**)*, Su(z)12* (**C**) and *Tip60* (**D**) gene region. Compared with input, anti-GFP immunoprecipitated chromatin from cyst cells showed E(Pc) enrichment at *E(z), Pc, Su(z)12* and *Tip60* genomic loci, peaks identified by peak calling were indicated by red lines below read density map and red box. The black line indicates average read density at chromosome.

Chapter 4

The study of histone inheritance patterns during asymmetric cell divisions of diverse *Drosophila* adult stem cells

INTRODUCTION

In eukaryotic nuclei, DNA is organized into nucleosomes by wrapping around histone octamers [2×(H3, H4, H2A, H2B)], which act as fundamental units to form the higher-order chromatin structure. Epigenetic mechanisms that alter chromatin structure, while preserving primary DNA sequences, contribute significantly to "cellular memory", which functions to maintain a particular cell fate throughout many cell divisions (Jacobs and van Lohuizen, 2002; Ringrose and Paro, 2004; Turner, 2002). Epigenetic regulation can occur at the level of DNA itself, such as DNA methylation, or at DNA-associated proteins, such as histones. The extensive post-translational modifications of histones have profound impacts on regulating gene expression (Berger, 2007; Fischle et al., 2003; Jenuwein and Allis, 2001; Schreiber and Bernstein, 2002; Turner, 2002). Adult stem cells may have a chromatin structure distinct from differentiated cells for their unique molecular characteristics, such as distinct gene expression patterns (Eun et al., 2010; Jaenisch and Young, 2008; Jenuwein and Allis, 2001) and alternative splicing patterns (Chepelev and Chen, 2013).

Among all epigenetic mechanisms, DNA methylation is the best characterized and has been identified to be responsible for epigenetic inheritance based on its semi-conservative manner of propagation (Bonasio et al., 2010; Martin and Zhang, 2007). It has also been shown that certain histone modifications at constitutively active genes, such as hyperacetylation of H3 and H4 or H3K4me2/3 and H3K79me2, are maintained in mitotic cells when global transcription is shut off, suggesting that histone modifications could serve as a molecular memory bookmarks to reestablish the transcriptional activation domain after mitosis (Kouskouti and Talianidis, 2005; Valls et al., 2005). In addition to active histone modifications, repressive histone modifications, such as H3K9me3, also remain associated with chromatin during mitosis (Fischle et al., 2005)

and is probably responsible for the faithful inheritance of heterochromatin structures (Irvine et al., 2006; Motamedi et al., 2004). However, it is unclear whether, as well as how histone modification patterns could be inherited in stem cells and/or reestablished in their differentiating daughter cells during ACD.

Based on existing knowledge and reagents, our lab developed a dual-color histone labeling system, similar to a previously reported strategy (Verzijlbergen et al., 2010). Using this system, preexisting histones can be precisely distinguished from newly synthesized histones because an irreversible DNA recombination induced the old-to-new histone switch (Tran et al., 2012). After the genetic switch in G2 phase GSCs (75% among all GSCs), we allowed them to undergo one round of the cell cycle because genome-wide incorporation of newly synthesized H3 occurs in S phase. We then traced the distribution of old versus new H3 in the GSC-GB (gonialblast) pair derived from the second mitosis of GSCs after the genetic switch. Strikingly, preexisting H3 was found to be asymmetrically distributed in GSCs, while newly synthesized H3 are enriched in GBs. As a control, we looked at the symmetrically dividing progenitor cells and found no evidence of asymmetric histone distribution. In addition, our lab demonstrated that phosphorylation of Threonine 3 of H3 (T3P) distinguishes preexisting H3 and newly synthesized H3. And a precise regulation of T3P is required for asymmetric segregation of H3 and also male germline activity (Xie et al., 2015). Using the same labeling strategy, we also studied the histone variant H3.3. In contrast to H3, H3.3 does not exhibit this asymmetric pattern during GSC divisions (Tran et al., 2012). It will be interesting to investigate whether this asymmetric histone distribution is a unique feature for asymmetric cell divisions of other adult stem cells.

Here, we explored segregation and distribution of existing old histone H3 GFP and newly synthesized H3 mKO during asymmetric cell division of different adult stem cells, including the

female germline stem cells (GSCs), male cyst stem cells (CySCs), neuroblasts (NBs) and intestinal stem cells (ISCs), using a similar experimental design (**Fig. 4-1**).

RESULTS AND DISCUSSIONS

Distribution of old and new histone H3 in female GSCs and Cystoblast (CB)

Female GSCs localized at the apical tip of ovarioles, where terminal filaments (TFs) and cap cells specify the microenvironment called niche (Kirilly and Xie, 2007; Lin and Spradling, 1993; Song et al., 2002). In vivo, female GSCs divide approximately once every 24 hours (Drummond-Barbosa and Spradling, 2001; Hsu et al., 2008; LaFever and Drummond-Barbosa, 2005; LaFever et al., 2010; Morris and Spradling, 2011). To allow the genome-wide incorporation of newly synthesized histone H3, the second S phase after heatshock was studied for histone distribution. Interestingly, fusome morphology changes during the cell cycle and can be used to pairs of GSC and CB (Ables and Drummond-Barbosa, 2013; de Cuevas and Spradling, 1998; Hsu et al., 2008; Salzmann et al., 2014) (**Fig. 4-2A**). Before heatshock, only H3 GFP, but not H3 mKO can be detected in the early germ cell (**Fig. 4-2B**), indicating the precise temporal control of our experimental system (**Fig. 4-1**). 45h AHS (after heat shock), H3 mKO is robustly expressed in germ cells and both H3 GFP and H3 mKO can be detected in GSC and CB pairs (**Fig. 4-2C**). Quantification of preexisting H3 GFP and newly synthesized H3 mKO in GSC and CB pairs revealed that old H3 GFP is distributed equally between female GSCs and differentiating CB (**Fig. 4-3A'**, **4-3B'**, **4-3C**). In most GSC and CB pairs, new H3 mKO is also equally distributed (**Fig. 4-3A", 4-3C**), while a subset exhibit more mKO in CB (**Fig. 4-3B", 4-3C**). Enriched mKO in a subset of CB, might be linked to the early entrance of S phase in these CBs, compared with GSCs.

The EdU incorporation assay would be a valuable strategy to detect the early DNA replication in CBs.

Overexpression of T3A (threonine to alanine) mutant caused excess germ cells in the ovary.

Besides the distribution of old and new histone H3 in S phase, potential segregation of histone H3 observed in male GSCs (Tran et al., 2012; Xie et al., 2015) was also explored in mitotic female GSCs. Phospho-T3 of H3 was used to detect GSCs undergoing mitosis. Interestingly, during prophase and prometaphase of female GSCs, old histone H3 GFP, instead of H3 mKO, co-localized more with mitotic marker T3P (**Fig. 4-4A, 4-4B**). At metaphase, both H3 GFP and H3 mKO are aligned at cell equatorial plate (**Fig. 4-4C**). Take together, these findings suggest that chromosomes carrying old and new H3 might have distinct congress characteristics. Considering the symmetric distribution of old and new H3 in S phase (**Fig. 4-3**), whether this mitotic segregation has a critical developmental role needs further study. Another evidence supporting the importance of segregation of new and old H3 is that overexpression of H3T3A in early germ cells leads to excess germ cells in 4.6% of *Nos>H3T3A* ovaries (N=87) (**Fig. 4-5 A-A"'**). The low penetrance of this phenotype might be caused by the low expression of UAS-driven transgene in the female germline. More interestingly, none of the excess germ cell phenotypes were induced in *Bam>H3T3A* ovaries (N=20), which have no H3T3A expression in GSCs, but robust H3T3A expression in CB and differentiating germ cells (**Fig. 4-5 B-B"'**). Similarly, no germ cell overproliferation phenotype was found for *Nos>H3S10A* ovaries (N=20), which harbor the H3S10A (serine to alanine) mutation with phosphorylation at Ser10 of H3 abolished (**Fig. 4-5 C-C"'**). These finding strongly support the crucial function of T3P in female GSCs specifically.

Segregation of preexisting and newly synthesized histone H3 in male CySCs

Drosophila male GSCs intimately assocaite with CySCs, which also undergo asymmetric cell division to produce two cyst cells (Cheng et al., 2011; Gonczy and DiNardo, 1996). Anaphase spindle repositioning is demonstrated to achieve the CySC asymmetric divisions (Cheng et al., 2011). We also explored potential segregation of preexisting and newly synthesized histone H3 during CySC divisions. Different from male GSCs, old and new H3 exhibited high colocalization in prophase (N=20) (**Fig. 4-6A**), metaphase (N=7) (**Fig. 4-6B**) and are symmetrically segregated in telophase (N=6) of CySCs (**Fig. 4-6C**). The different segregation pattern of old and new H3 in CySCs and neighboring GSCs might be related to distinct differentiation programs of GSCs and CySCs lineages. Unlike germline cells, CySCs only divide once and differentiating cyst cells never divide again. In the absence of germ cells, cyst cells continue to proliferate and can even switch to the hub cell fate (Gonczy and DiNardo, 1996), implying the essential role of germ cells in regulating proliferation and cell fate of CySCs lineage. Thus, it is possible that alterations of intrinsic epigenetic signature between CySCs and cyst cells are not as critical as in GSCs and GBs. In addition to the distinct H3 segregation pattern, mitotic marker H3T3P can still be detected robustly in telophase of CySCs (**Fig. 4-6C**), but not in GSCs (Xie et al., 2015). This suggests the temporal regulation of phosphorylation of H3T3 in mitosis is lost in CySCs. It is interesting to further study how H3T3P persists in CySCs while diminishing in GSCs as well as characterizing the dynamic regulation of this mitotic modification in two adult stem cell lineage.

Distribution of old and new histone H3 in intestinal stem cells (ISCs)

In addition of CySCs, another well-studied somatic stem cell is ISC, which is found in the Drosophila midgut (mgISC) (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006) and hindgut (hgISC) (Takashima et al., 2008). Upon asymmetric cell division, mgISCs produce self-renewal ISCs and quiescent enteroblasts (EB cells), which further differentiate to enterocytes

(ECs) or enteroendocrine (EE) cells. Notch signaling plays important roles in mgISCs differentiation. Although both mgISC and EBs, as well as early differentiated cells, express the Notch receptor, expression of ligand Delta in mgISCs activate Notch signaling in daughter cells to promote differentiation (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006) (**Fig. 4-7A**). We applied escargot (esg) -Gal4, expressed in both ISCs and EB cells (Micchelli and Perrimon, 2006; Zeng et al., 2010), to drive histone H3 expression (**Fig. 4-1**). Delta staining was used to identify ISCs (**Fig. 4-7B-C**) and Notch signal reporter Su(H)Gbe-LacZ was applied to detect EBs (**Fig. 4-7D-E**). We found equally distributed old H3 GFP (**Fig. 4-7C', E'**) and new H3 mKO (**Fig. 4-7C"**) between ISCs and EBs. Consistent with this distribution, symmetric segregation of H3 GFP and new H3 mKO was also seen in telophase of ISCs (**Fig. 4-7F-F"'**). Interestingly, it is likely that not all H3 GFP and H3 mKO colocalize in telophase (**Fig. 4-7F"'**). More mitotic data are required to further show the potential H3 GFP and mKO enriched domain in mitotic ISCs.

Distribution of old and new histone H3 in NBs

During Drosophila neurogenesis, NBs undergo asymmetric cell divisions to generate diverse neurons and glial cells (Doe, 2008; Saini and Reichert, 2012; Yu et al., 2006). Recent studies have found there are two types of NBs (Bayraktar et al., 2010; Bello et al., 2008; Boone and Doe, 2008; Bowman et al., 2008). After asymmetric cell division of type I NBs, the larger apical daughter cell functions as an NB, while the smaller basal daughter cell becomes a ganglion mother cell (GMC), which undergoes divisions to generate postmitotic neurons or glia (**Fig. 4-8A**). Different from type I NBs, differentiated daughter cells of the type II NB (**Fig. 4-8B**) can function as intermediate neural progenitor cells (INPs) and they can further divide between four and eight times. We mainly analyzed histone H3 distribution in type I NBs using Insc-Gal4 (Betschinger et al., 2006). Atypical protein kinase C (aPKC) (Rolls et al., 2003) is maintained in NBs and was

used to detect NBs side during mitosis (**Fig. 4-8C**). Due to the 2-hour cell cycle of NBs, mitotic NBs at 3.5 hours after heatshock were studied to investigate the potential segregation of H3 GFP and mKO. However, no detectable H3 mKO was observed (**Fig. 4-8D**), although H3 mKO eventually expressed at detectable levels 20 hours after heatshock (**Fig. 4-8E**). This suggests after irreversible DNA recombination (**Fig. 4-1**)**,** it takes more than 3.5 hours to detect H3 mKO fluorescence. H3 GFP localization relative to H3T3P was further studied in prophase (**Fig. 4-9A**), prometaphase (**Fig. 4-9B-C**), metaphase (**Fig. 4-9D**) and telophase (**Fig. 4-9E**). Interestingly, H3 GFP positive, H3T3P negative domains were found in prometaphase and metaphase. In addition, in telophase of NBs, there seems to be more H3 GFP in the stem cell side, compared with the GMC side. However, more telophase data and quantifications are required to demonstrate the potential asymmetric histone H3 segregation. In addition, photoswitchable fluorescent protein Dendra could be applied in the future to study both old and new histone segregation during NBs divisions.

MATERIALS AND METHODS

Heatshock regime:

2 hours heatshock in a water bath at 37 °C were applied to newly eclosed adult female flies *hs-flp; Nos-Gal4/UASp-FRT-H3-GFP-PolyA-FRT-H3- mKO*, male flies *hs-flp; Tj-Gal4/UASp-FRT-H3-GFP-PolyA-FRT-H3- mKO*, female flies *hs-flp; esg-Gal4/UASp-FRT-H3-GFP-PolyA-FRT-H3- mKO,* female flies *hs-flp/Su(H)GBE-LacZ; esg-Gal4/UASp-FRT-H3-GFP-PolyA-FRT-H3-mKO.* These flies were recovered at 29°C for 42-45 h (*Nos>H3*), 18-20 h (*Tj>H3*), 28-30 h (*esg>H3*) until dissection. Larval flies *hs-flp; Insc-Gal4/UASp-FRT-H3-GFP-PolyA-FRT-H3-mKO* were heat shocked for 1-1.5 hour and recovered at 29°C for 2-3 hours until dissection.

Immunostaining

Drosophila testes were dissected and stained with pH3 (Rabbit, 1:200, Millipore 05-746R), mKO (mouse, 1:500, M168-3, MBL), FasIII (Mouse, 1:100, DSHB, 7G10). Drosophila ovaries were dissected and immunostained with Vasa (rabbit, 1:200, Santa Cruz, sc-30210), pH3, mKO, α-spectrin (Mouse, 1:50, DSHB, 3A9), N-Cadherin (rat, 1:20, DN-EX#8, DSHB). Guts were dissected and stained with pH3, mKO, β Galactosidase (rabbit, 1:10000, Cappel) as previous described (Li et al., 2013). Delta (mouse, 1:50, C594.9B, DSHB) staining was performed using methanol heptane fixation method described before (Lin et al., 2008). DSS (dextran sulfate sodium) treatment was applied to induce ISC divisions (Amcheslavsky et al., 2009). Larval brains were dissected following the protocol used before (Wu and Luo, 2006) and stained with aPKC (rabbit, 1:1000, Santa Cruz Biotech), pH3.

FIGURES

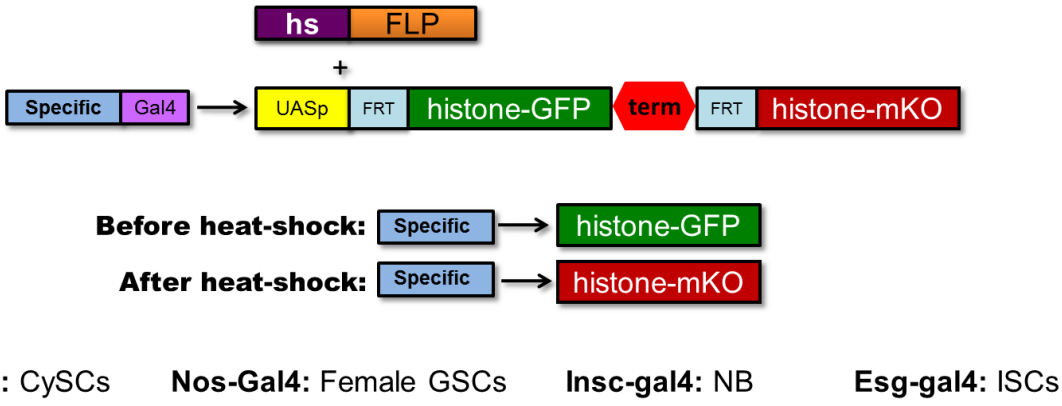

Figure 4-1: Experimental design. Hs-Flp: yeast recombinase controlled by the heatshock promoter. Nanos (nos)-Gal4: early germ cell driver. Traffic Jam (Tj)-Gal4: an early cyst cell driver. Inscuteable (Insc)-Gal4: type I and II NBs and INPs. Escargot (Esg)-Gal4: ISCs and EB cell driver.

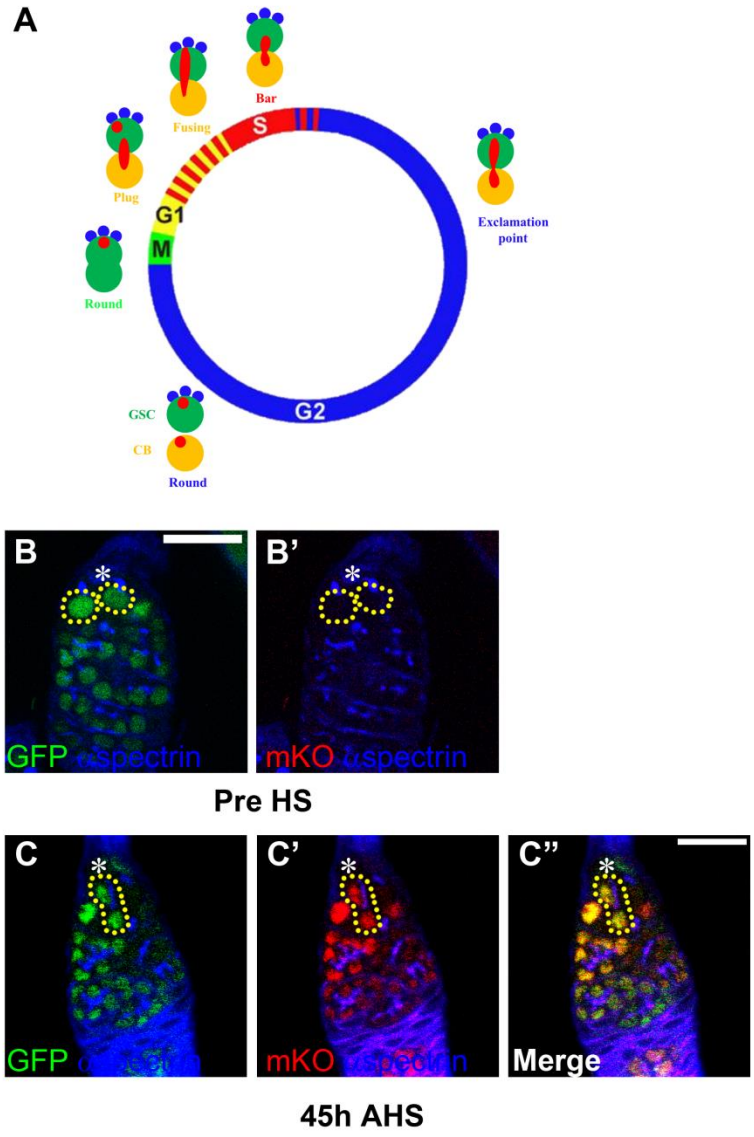

Figure 4-2: "Old" histone H3 GFP and "New" histone H3 mKO in female germline cells. (**A**) Dynamic fusome morphology in different cell cycle stages. Modified from (Ables and Drummond-Barbosa, 2013). (**B**) Before heatshock, H3 GFP expression in early germ cells. No H3 mKO is detected. Yellow outline: GSCs. (**C**) H3 GFP and H3 mKO expression in early germ cells 45h after heatshock. Yellow outline: GSC and CB resulting from one asymmetric cell division. Asterisk: cap cells. Scale bar: 20μm.

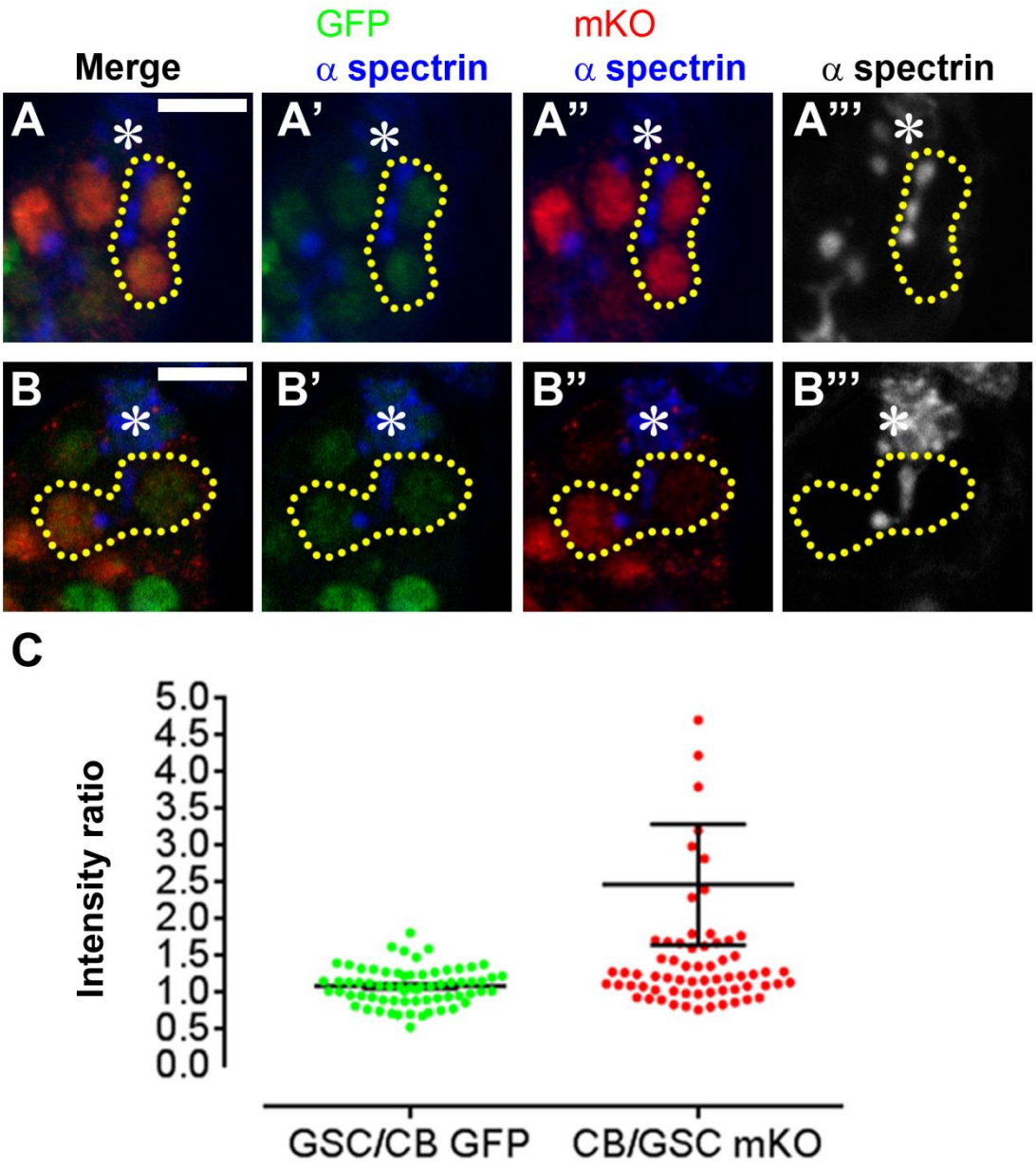

Figure 4-3: Distribution of "old" H3 GFP and "new" H3 mKO in GSC and CB. H3 GFP (A', B') and H3 mKO (A") are distributed equally between GSCs and CBs. A subset population of CB have more H3 mKO, compared with GSCs (B"). (**A-B**) Images are projections of multiple stacks. Asterisk: cap cells. Scale bar: 10μm. (**C**) Quantification of intensity ratio between GSC and CB.

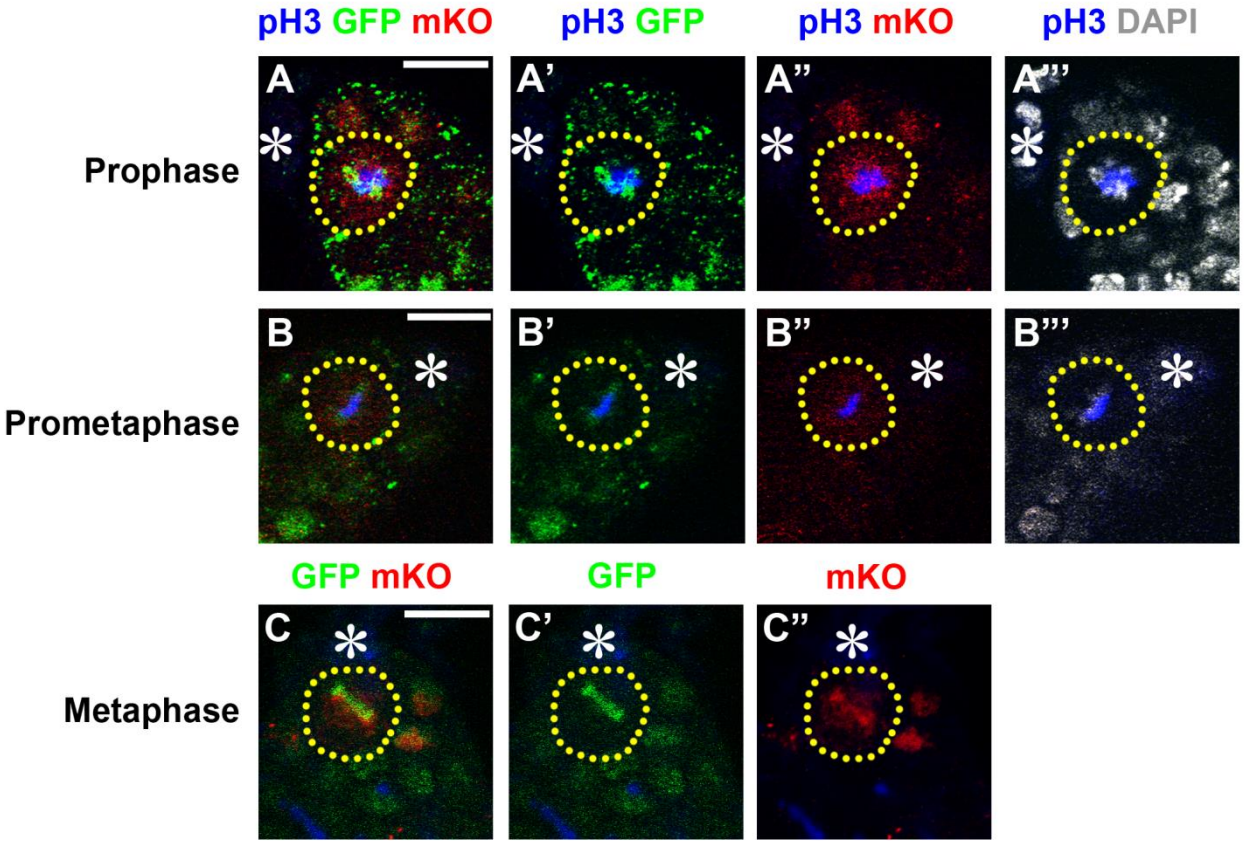

Figure 4-4: Segregation of "old" H3 GFP and "new" H3 mKO in prophase (A-A"'), prometaphase (B-B"') and metaphase (C-C") of GSCs in the ovary. Asterisk: cap cells. Scale bar: 10μm.

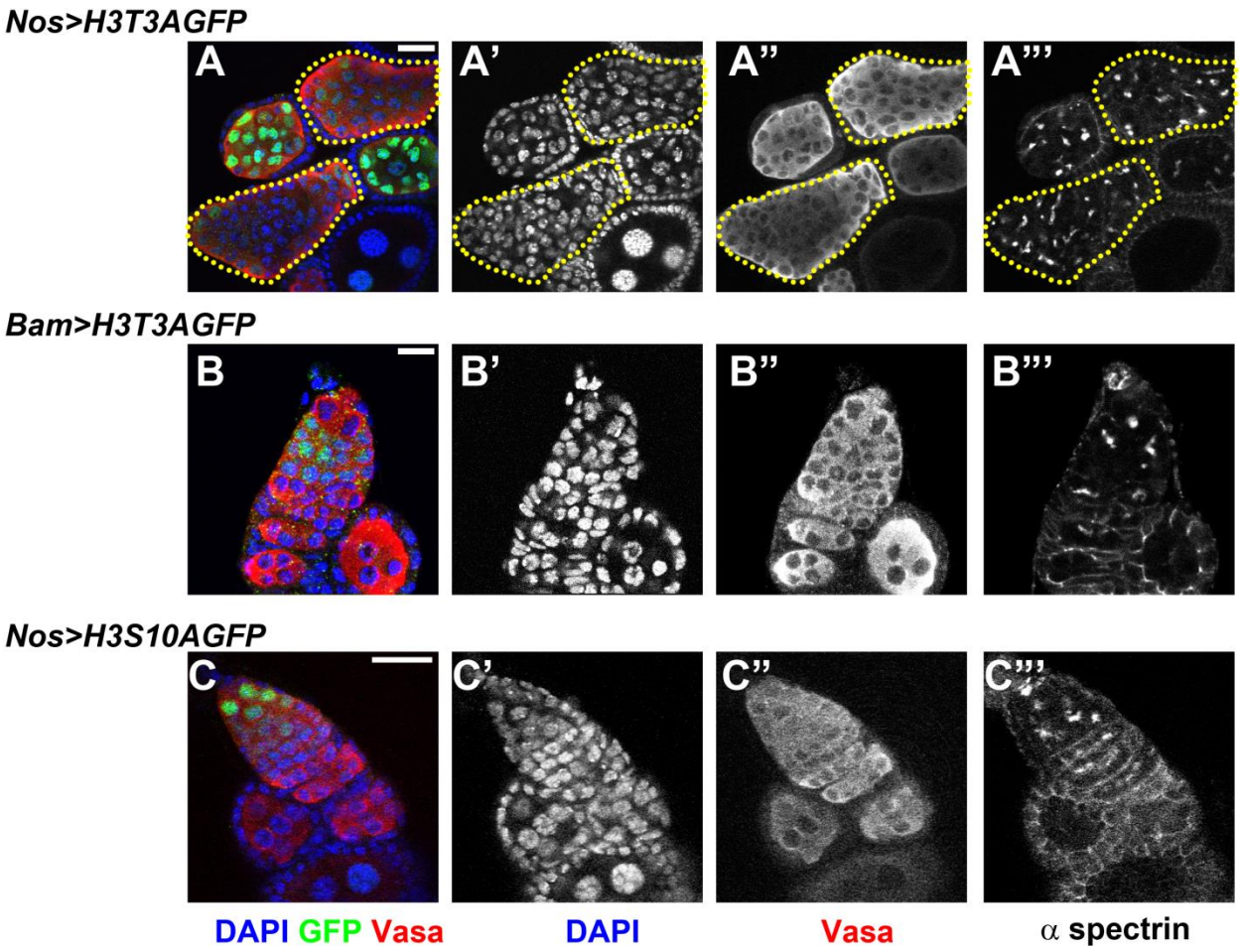

Figure 4-5: Germline overproliferation is induced in *Nos>H3T3AGFP* ovary (A-A"'), not in *Bam>H3T3AGFP* (B-B"') and *Nos>H3S10AGFP* (C-C"') ovary. Immunostaining with germ cell marker Vasa and fusome marker α spectrin. (A-A"') yellow outline: overpopulated germ cells. Scale bar: 20μm (A, C), 10μm (B).

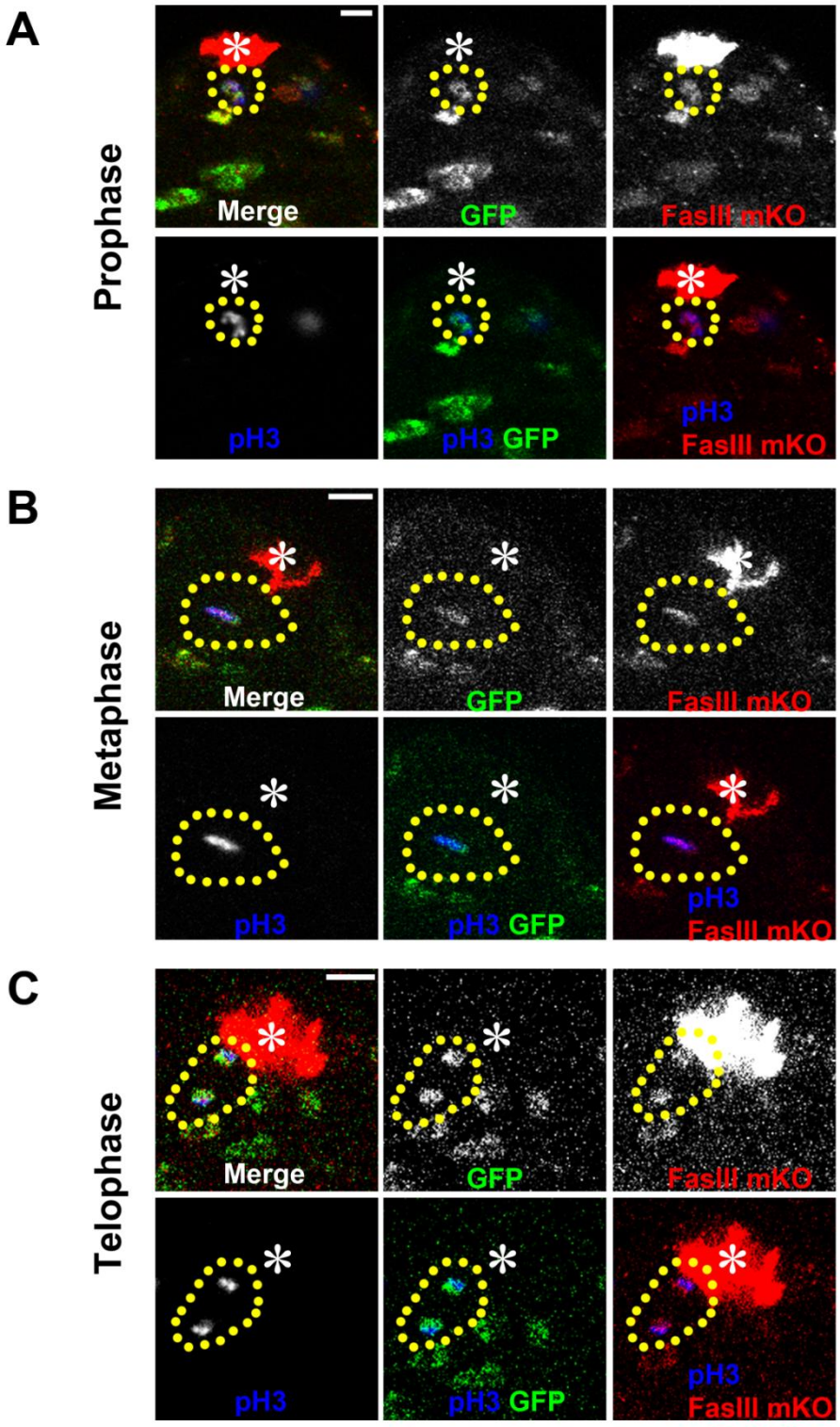

Figure 4-6: Segregation of "old" H3 GFP and "new" H3 mKO in prophase (A), metaphase (B) and telophase (C) of CySCs. Asterisk: Hub cells (labeled by FasIII). Scale bar: 5μm.

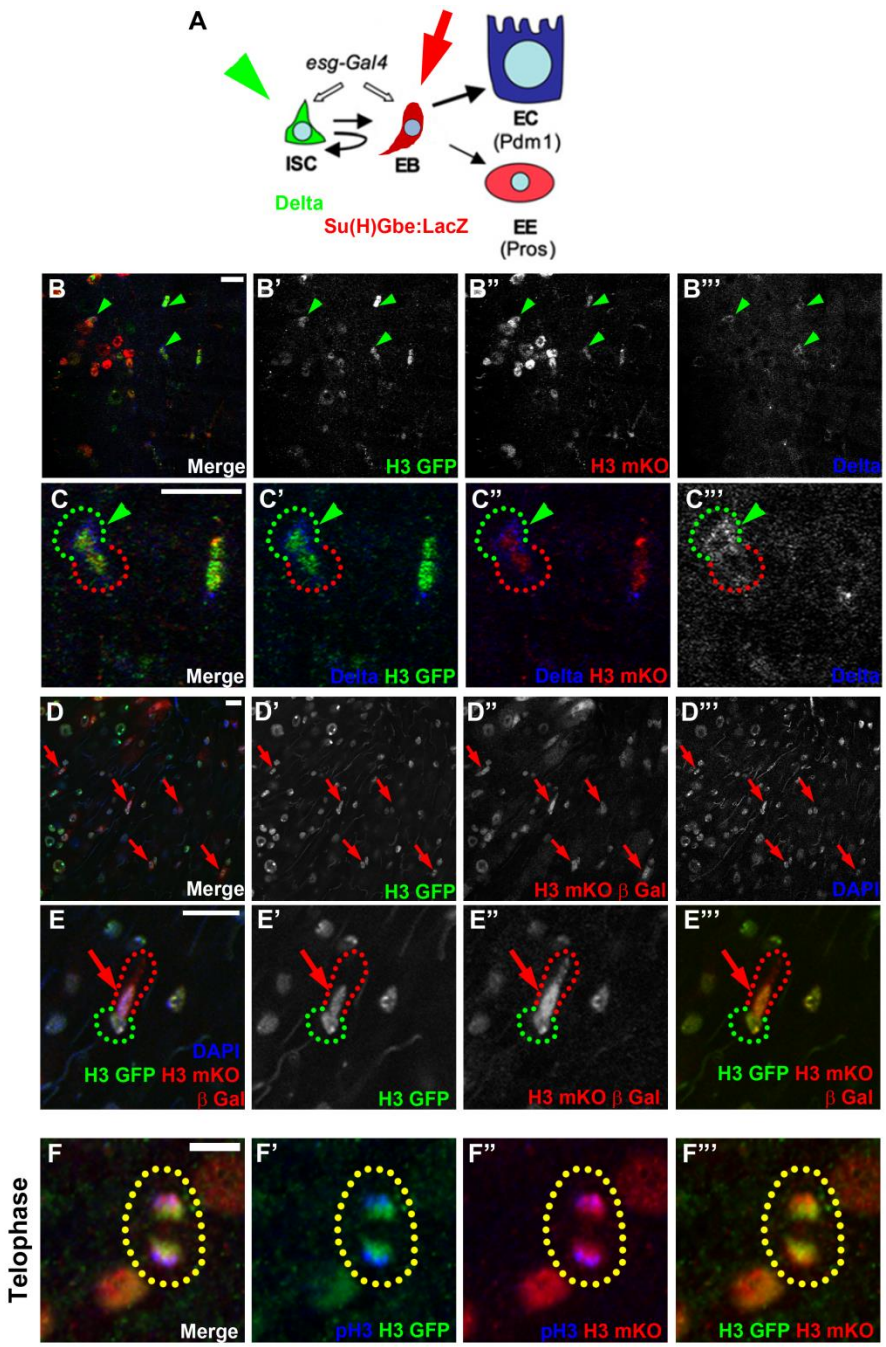

Figure 4-7: Distribution of "old" H3 GFP and "new" H3 mKO in ISC and EB. (**A**) Diagram of asymmetric cell division of ISC. ISC is enriched with Delta, while EB has high Notch activity. Su(H)-Gbe lacZ: a transcription reporter of Notch signaling. (**B-C**) Immunostaining with Delta identifies ISC (green arrowheads) and neighboring EB. (**D-E**) Immunostaining with β Gal

identify EBs (red arrows), which have high Notch activity, and neighboring ISCs. (**F-F"'**) Segregation of "old" H3 GFP and "new" H3 mKO in telophase of ISCs. Scale bar: 5μm.

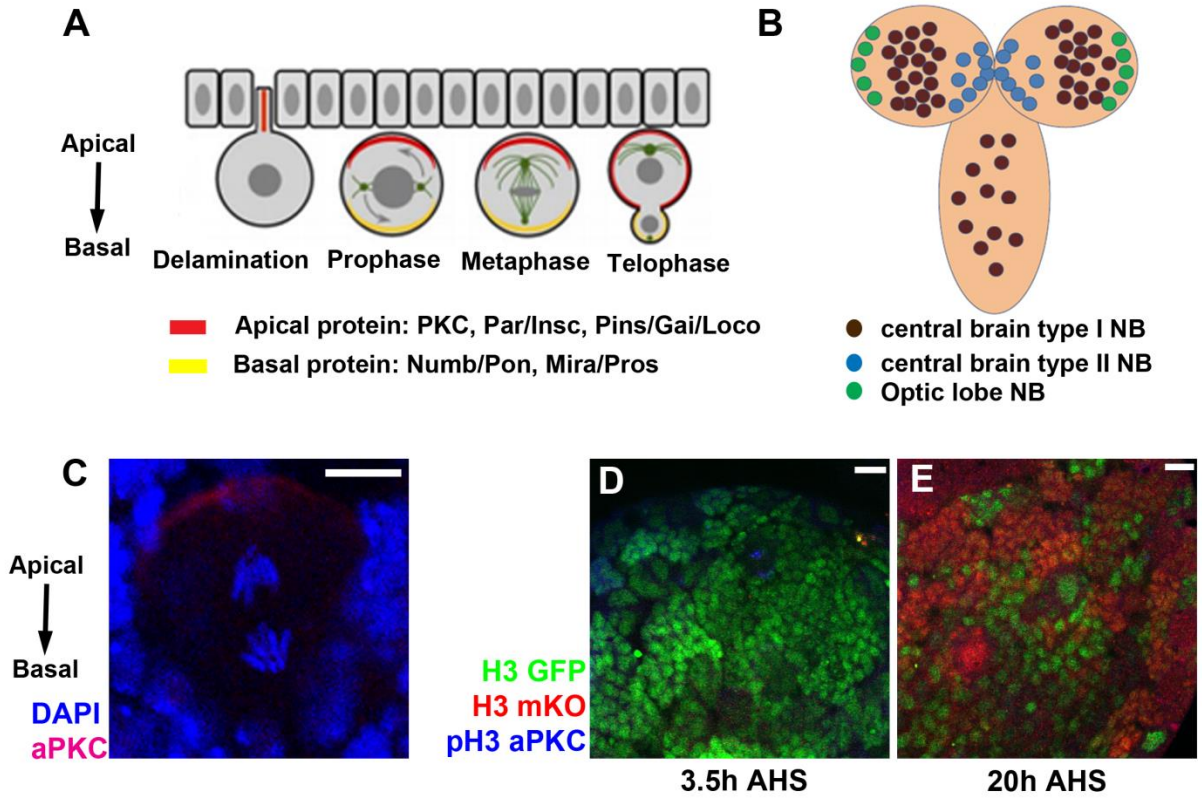

Figure 4-8: Asymmetric cell division of NB. (**A**) Diagram of asymmetric cell division of NB and polarized protein distribution. Adapted from (Yu et al., 2006). (**B**) Distribution of different types of NBs. Adapted from (Saini and Reichert, 2012). (**C**) Dividing NB immunostained with atypical protein kinase C (aPKC). Scale bar: 5μm. Expression of H3 GFP and H3 mKO 3.5 hours after heatshock (**D**) and 20 hours after heatshock (**E**) in neurons. Scale bar: 10μm.

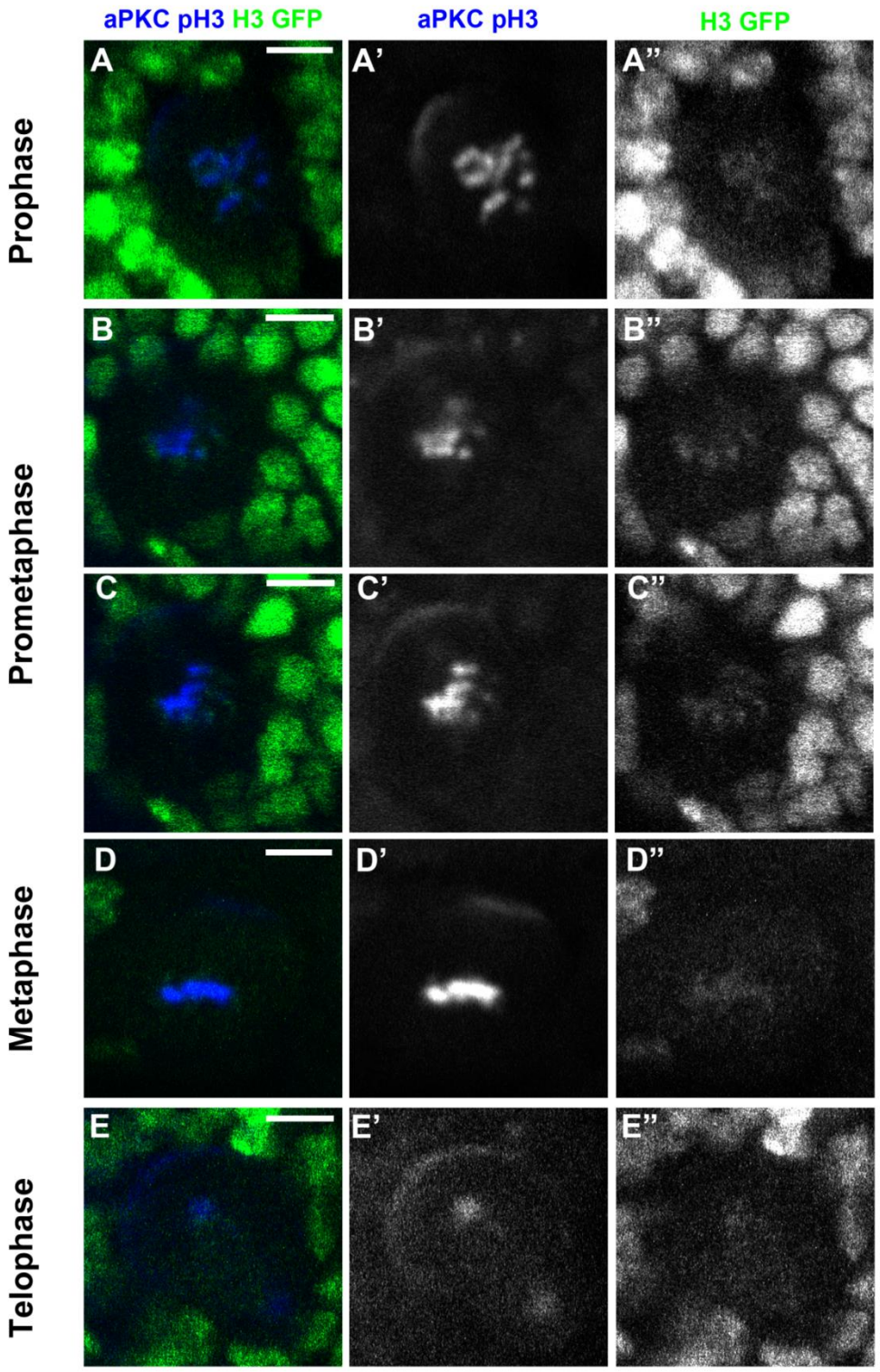

Figure 4-9: Distribution of H3 GFP in prophase (A-A"), prometaphase (B-C"), metaphase (D-D") and telophase (E-E") of NB. Immunostaining with aPKC and H3T3P. Scale bar: 5μm.

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Zoller, R., and Schulz, C. (2012). The Drosophila cyst stem cell lineage: Partners behind the scenes? Spermatogenesis *2*, 145-157.

Curriculum Vitae

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Education

2016 Ph.D., Molecular and Developmental Biology
Johns Hopkins University, MD

2010 B.S., Cellular Biology,
University of Science and Technology of China (USTC), Hefei, China

Research Experience

2011-present Doctoral Thesis research
Department of Biology
Johns Hopkins University
Advisor: Dr. Xin Chen
Title: *Study a novel chromatin factor E(Pc) in stem cell lineages*
Summary: I use various approaches including genetics, genomics and cell biology to study functions of the chromatin factor E(Pc) in stem cell maintenance and differentiation. I found E(Pc) in germ cells promotes germline stem cell differentiation and also maintains genome integrity. Moreover, E(Pc) acts non-cell autonomously to maintain the germline activity and prevent the germline-to-soma transition.

2009-2010 Undergraduate research
School of Life Sciences
University of Science and Technology of China
Advisor: Dr. Xuebiao Yao
Title: *Function of the GAP protein ACAP4 in cell migration*
Summary: Biochemical and cell biological techniques were used to study roles of the GTPase-Activating Factor ACAP4 in cell migration. Multiple phosphorylation sites of ACAP4 upon EGF stimulation were identified using mass spectrometry.

Teaching and Supervisory Experience

2011-2012 Teaching Assistant for undergraduate laboratory class
Genetics, Developmental Biology

2011-2016 Supervise 3 rotations students, 3 undergraduate students
Help students develop the projects, design experiments and analyze data

Honors and Awards

2009	Outstanding Student Scholarship (Grade 1), USTC, China
2009	National Endeavor Scholarship (Fellowship), USTC, China
2008	Outstanding Student Scholarship (Grade 1), USTC, China
2007	Advanced Individual of Social Practice, USTC, China
2007	Outstanding Student Scholarship (Grade 2), USTC, China

Professional Membership

2012- present Genetics Society of America Membership

Oral Presentations

1. Enhancer of Polycomb represses transcription of Cyclin B during male germ cell differentiation. (2016)
57th Annual Drosophila Research Conference, Orlando, FL.

2. A novel chromatin factor Enhancer of Polycomb acts in somatic cells to maintain germ cell identity and activity in Drosophila adult testis. (2015)
56th Annual Drosophila Research Conference, Chicago, IL.

3. E(Pc) coordinates differentiation of multiple stem cell lineages. (2015)
17th Annual JHU CMDB retreat, St Michaels, MD.

Poster Presentations

1. Enhancer of Polycomb coordinates multiple signaling pathways to promote both cyst and germline stem cell differentiation in Drosophila adult testis. (2016)
Epigenetics & Chromatin, New York, NY.

2. Enhancer of Polycomb maintains germ cell identity and activity in Drosophila adult testis. (2014)
Mid-Atlantic SDB Meeting, Baltimore, MD.

3. Investigate roles of Enhancer of Polycomb in both germline and somatic gonadal cells during Drosophila spermatogenesis. (2013)
Stem cell Biology, New York, NY.

Publications

1. **Feng, L.**, Shi, Z., Chen, X. (2016). Enhancer of Polycomb maintains germline activity and genome integrity in Drosophila testis. ***Nature Communications*** (under revision).

2. **Feng, L.**, Shi, Z., Chen, X. (2016). Enhancer of Polycomb coordinates multiple signaling pathways to promote both cyst and germline stem cell differentiation in Drosopila adult testis. ***PLoS Genetics*** (minor revision).

3. Eun, S.H.*, **Feng, L.***, Chen, X. (2016) E(z) is required for germ cell dedifferentiation in Drosophila testis. (* equal contribution). (in preparation).

4. Lim, C., Gandhi, S., Biniossek, M.L., **Feng, L.**, Schilling, O., Urban, S., and Chen, X. (2015). An Aminopeptidase in the Drosophila Testicular Niche Acts in Germline Stem Cell Maintenance and Spermatogonial Dedifferentiation. ***Cell Reports*** *13*, 315-325.

5. Cheong, F.K., **Feng, L.**, Sarkeshik, A., Yates, J.R., 3rd, and Schroer, T.A. (2014). Dynactin integrity depends upon direct binding of dynamitin to Arp1. ***Molecular Biology of the Cell*** *25*, 2171-2180.

6. **Feng, L.**, and Chen, X. (2015). Epigenetic regulation of germ cells-remember or forget? ***Current Opinion in Genetics & Development*** *31*, 20-27.

7. Tran, V.*, **Feng, L.***, and Chen, X. (2013). Asymmetric distribution of histones during Drosophila male germline stem cell asymmetric divisions. ***Chromosome Research*** *21*, 255-269. (* equal contribution).